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MORPHOLOGICAL AND PHYSIOLOGICAL ADAPTATIONS OF PROSTHECATE
BACTERIA TO GROWTH IN LOW NUTRIENT ENVIRONMENTS

PHILIP MORGAN B.Sc. (BATH)

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University of Warwick,
Department of Biological Sciences.

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SUMMARY

Prosthecae bacteria are usually observed in oligotrophic (low nutrient) environments and have been proposed as "model" oligotrophic bacteria since they possess a number of adaptations for growth under very low nutrient conditions. This project was designed to study selected prosthecae bacteria and attempt to relate their ecology to physiological and morphological adaptations, in particular the production of a motile swarmer cell stage.

Ecological investigations showed that the prosthecae bacteria were ubiquitous in the oligotrophic fresh water environments examined. A variety of morphological types were observed in populations which also included large numbers of morphologically "typical" cells, demonstrating that there exists a large variety of oligotrophic bacteria. The responses of these bacteria to increasing nutrient concentrations suggested that currently accepted definitions of oligotrophy are in need of reconsideration. Caulobacter crescentus CB15, Hyphomicrobium X and Rhodomicrobium vannielii Rm5 were studied in detail in both batch and continuous culture with respect to nutrient effects on cell morphology and cell type expression. All three organisms produced elongated prosthecae with increasing nutrient stress under both carbon and phosphate-limited conditions, an observation somewhat in contradiction with reports suggesting that prosthecae function as specialised phosphate uptake sites. The production of swarmer cells was enhanced under conditions of nutrient stress, supporting the proposition that swarmer cells function as specialised survival and dispersal cells.

R. vannielii was chosen for investigations into the intermediary metabolism of prosthecae bacteria with respect to adaptations to oligotrophy and differential cell type expression. Unlike other Rhodospirillaceae this organism was shown to possess an incomplete tricarboxylic acid (TCA) cycle under anaerobic conditions broken at 2-oxoglutarate dehydrogenase and lacked NADH oxidase activity but these enzymes were present under aerobic conditions. Of the glyoxylate shunt enzymes, malate synthase activity was detected but isocitrate lyase was absent. The TCA cycle enzymes, Ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) and phosphoenolpyruvate carboxylase possessed similar activities and inhibitor patterns in both swarmer and prosthecae cells and therefore the physiological adaptations of the swarmer cells remain largely unknown.

This work has demonstrated the success and ubiquity of prosthecae bacteria in the oligotrophic fresh water ecosystem although it must be emphasised that there exist a large number of non-prosthecae bacteria in these environments. The roles of the prosthecae and swarmer cells in this competitiveness are as yet not fully clear but appear to be of importance in view of observed responses to nutrient limitation.

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This thesis is dedicated with love to my parents and to Ruth in appreciation of their continual help and support.

DECLARATION

I declare that all work in this thesis, except where specifically stated, was original research performed by myself under the supervision of Dr. C.S. Dow and that none of this work has previously been submitted for any degree. All sources of information have been acknowledged by means of references.

P. Morgan

PHILIP MORGAN

ABBREVIATIONS

A	Absorbance
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CoA	Coenzyme A
cpm	Counts per minute
D	Dilution rate
DCPIP	Dichlorophenolindophenol
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DTNB	5,5'-biethiol (2-nitrobenzoic acid)
EC	Enzyme commission number
EDTA	Ethylenediaminetetraacetic acid
g	Gravitational force
h	Hour
HM	<u>Hyphomicrobium</u> medium
HMB	Hutner's minimal medium plus glucose
min	Minute
MOPS	3-[N-morpholino]propanesulphonic acid
M9	Minimal salts medium (Lark <u>et al.</u> , 1963)
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADH	Reduced form of NAD
NADPH	Reduced form of NADP
PEP	Phosphoenolpyruvate
PGA	Phosphogluconate

PHB	Poly- β -hydroxybutyrate
PP _i	Inorganic polyphosphate
PM	Pyruvate-malate medium
PMS	Phenazine methosulphate
PMYE	Pyruvate-malate-yeast extract medium
POC	Particulate organic carbon
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RUBISCO	Ribulose-1,5-bisphosphate carboxylase-oxygenase (EC 4.1.1.39)
RuBP	Ribulose-1,5-bisphosphate
sp./spp.	Species (singular/plural)
t _d	Doubling time
TCA	Tricarboxylic acid (cycle)
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
v/v	Volume to volume
w/v	Weight to volume
μ	Specific growth rate
μ_{max}	Maximum specific growth rate
μ_{min}	Minimum specific growth rate

PART 1 - INTRODUCTION

"Seventy percent of the surface of the biosphere and more than 90% of its volume is occupied by aquatic biotypes [and] most of these ecosystems can be considered extreme habitats since they approach in their nutrient content the lowest possible limits necessary to sustain organisms."

Shilo, 1980.

"Much can be learned from a study of these 'unusual' (budding) organisms; 'unusual' here should be understood as 'exceptional to the rule'. These exceptions, the unusual bacteria, may well hold explanations to secrets that cannot even be visualised from studying the well known, 'usual' organisms."

Hirsch, 1974.



ABOVE - Esthwaite Water, Cumbria, an oligotrophic lake, and the names of the three "oligotrophic" prosthacate bacteria employed in this study.

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1.1) GENERAL OVERVIEW - OLIGOTROPHY AND MICROBIAL ECOLOGY

1.1.1) The legacy of Robert Koch

It has been stated (Bull & Quayle, 1982) that as a consequence of the work of Robert Koch published in 1881, the science of microbiology has developed with an oversimplistic view of the behaviour of microorganisms in the natural environment. The legacy of Koch has been an emphasis on the use of monoculture, batch growth, single substrate utilisation and experimental homogeneity. It is now clear that nearly all microbial activity in the natural environment occurs with mixed populations utilising a range of substrates (Parkes, 1982), in systems where "substrate limitation is undoubtedly the rule" (Veldkamp, 1976). Furthermore, most experimental microbial ecology has been performed in either batch or homogeneous continuous culture and has neglected the existence of the spatial and temporal heterogeneities that exist in the natural ecosystem in such forms as physico-chemical gradients and temporal variations in nutrient supply (Wimpenny, 1985). It has only been comparatively recently that the complexities of the natural environment and the restrictions imposed on the microorganisms therein have gained widespread attention. As a consequence of this interest, the ubiquity of extreme low nutrient environments and the importance of the indigenous organisms in the cycling of nutrients on the global scale (Shilo, 1980) are now receiving their due degree of appreciation.

1.1.2) The low nutrient natural environment

On a global scale, the overwhelming majority of natural microbial habitats contain extremely low levels of organic and inorganic nutrients. Merely by calculating the volume of the worlds oceans of more than 1000m depth, it is possible to account for approximately 90% of the volume of the biosphere and 97.6% of the total volume of sea water, all of which, with the exceptions of the photic primary production zone and highly localised nutrient-rich zones, contain extremely low concentrations of nutrients (Jannasch, 1984). Furthermore, by additionally considering the rest of the nutrient-poor sea, fresh water and soil environments, it is clear that the majority of bacteria in the natural environment exist under conditions of extreme nutrient limitation. However, it cannot be overemphasised that in the natural environment microbial activity will not be restricted merely by one nutrient nor by nutrient limitation alone (Morgan & Dow, 1986).

Hirsch (1979) defined the low nutrient ecosystem as "one in which the the flux of nutrients across the ecosystem is so small relative to the volume of that ecosystem, that, averaged over time and space and allowing for heterogeneities within the system, the effective turnover rate is low", a definition which makes many of the salient points necessary to any consideration of the microbial ecology of the environments. It cannot be overemphasised that in any nutrient-poor system the organisms therein are functioning as members of communities in a heterogeneous environment. Over

the years many attempts have been made to classify microbial environments into types on the basis of their trophic status and to classify the bacteria therein in a similar manner. Currently the concept used by most microbiologists is that of oligotrophy which, in the sense most widely accepted at present, makes a large number of oversimplifications and assumptions.

1.1.3) The concept of oligotrophy

The division of microorganisms into groupings on the basis of their responses to nutrient levels in situ has long been proposed. Winogradsky divided soil microorganisms into two groups based on their behaviour in the natural environment (Kuznetsov et al., 1979) . The term autochthonous was applied to those organisms which are found in soil under all conditions and maintain themselves at a constant rate in the environment. The term zymogenous was applied to those microorganisms, characteristically alien to the environment, which rapidly grow in response to a, typically introduced, nutrient which is readily available, but cannot maintain themselves in the environment under normal conditions. In addition the r-strategists and K-strategists of ecological theory resemble zymogenous and autochthonous organisms respectively (Koch, 1979).

The term oligotrophic has been widely used in ecology with the meaning of a general low level of nutrients in an environment, the term eutrophic being used for a comparatively nutrient-rich system. Kuznetsov et al.

(1979) extended the definition of the term oligotrophic to cover bacteria capable of, and presumably specialised for, growth in nutrient-poor environments. Oligotrophic bacteria were classified as those bacteria which, on first isolation from the natural environment, are capable of growth on media containing 1-15mg organic carbon l^{-1} and that grow on such media on subsequent cultivation, although they may or may not be able to grow on media containing higher levels of carbon. Poindexter (1981a; 1981b) endorsed and further extended this definition by defining a second group of bacteria, the copiotrophs, which are unable to grow on such low carbon media and require a nutrient supply "100 times higher" for survival than that needed for oligotrophic bacteria. In addition she subdivided the "oligotrophs" into two groups, the obligate and facultative oligotrophs, the former being those forms that are incapable of growth in "high nutrient" media. Despite the obvious and highly significant flaws in these definitions, there being a reliance on cultivation and media selection, with a consequent subjective bias, and the absence of any consideration of in situ environmental factors and heterogeneities, most subsequent work has not criticised these definitions other than to alter absolute dividing lines. For example, Ishida & Kadota (1981) and Ishida et al. (1980) divided the oligotrophs from the copiotrophs at a level of 1mg organic carbon l^{-1} in their medium but at least attempted to introduce an environmental slant to their definitions by defining copiotrophs as those organisms

able to grow at surfaces in situ whereas oligotrophs cannot. The basing of the concepts of obligate and facultative oligotrophs on the inability of organisms to grow on nutrient-rich media has been questioned by Martin & MacLeod (1984), who demonstrated that the responses of bacteria to varying levels of organic carbon are dependent on the carbon source used. Many organisms incapable of growth on "high" concentrations of the substrate normally employed for such studies, bacteriological peptone, will grow on identical carbon concentrations of defined substrates. Furthermore, the use of defined or man-made carbon sources may yield artefactual results. In the low nutrient aquatic environment the bulk of carbon compounds will be of algal and cyanobacterial origin. These have been demonstrated to select for a different "type" of oligotroph in chemostat experiments than those forms normally isolated on peptone media (Bell, 1984). The former were observed to resemble more closely the archetypal oligotroph (Hirsch, 1979) in that they possessed high affinity, constitutive nutrient uptake systems and were catabolically versatile.

A number of workers have proposed that organisms commonly found in low nutrient environments in some way represent model oligotrophs (e.g. Arthrobacter and Caulobacter; Poindexter, 1981a) and that these possess specialised properties for growth in such environments (Table 1.1; Hirsch, 1979; Shilo, 1980). Whilst it cannot be doubted that a range of physiological properties can be of benefit to organisms under conditions of extreme nutrient limitation

TABLE 1.1 - Some supposed properties of model obligate oligotrophs (Hirsch, 1979; Poindexter, 1981a).

Obligately aerobic.

Constitutive nutrient uptake systems.

High affinity and broad specificity uptake systems.

Accumulation of substrate reserves.

Low endogenous metabolic rate.

Efficient use of energy.

Catabolically versatile via inducible enzyme systems.

Potential for "sensing" of environment.

(see below, Section 1.6), it would appear that most of these properties and an ability to grow at extremely low nutrient concentrations are evident in a range of bacteria, including the "model copiotroph" Escherichia coli (Koch, 1979).

Although it has been suggested that intracellular amino-acid levels (Stepanovich et al., 1984) or the absence of metabolic mechanisms for dealing with oxygen radicals at "fast" growth rates (Kuznetsov et al., 1979; Poindexter, 1981a) may be at least partially responsible for the nutrient sensitivity of obligate oligotrophs, there is no firm evidence of any physiological properties that could explain the proposed behaviour of such organisms.

In this thesis I will use the terms oligotrophic and oligotrophy in their broad ecologically-based meanings as a

convenient label for referring to types of environment and organisms and not in the stricter contexts of in vivo responses nor as firm defined types of organisms. Indeed, I propose that, at present, the terms should solely be employed in the ecological sense employed by Hirsch (1979).

1.1.4) Oligocarbophily

The term oligocarbophily applies to bacterial growth in carbon-free media where the organisms utilise dissolved volatile organic carbon compounds of atmospheric origin. Hirsch (1964) identified a range of volatile substrates potentially available to Nocardia and related organisms, including hydrocarbons, alcohols, aldehydes, ketones, fatty acids and aromatics. Chesney et al. (1985) have demonstrated the mineralisation of phenol at concentrations as low as 1 ng ml^{-1} by natural fresh water assemblages of bacteria and Geller (1983) has shown that the growth rate of Pseudomonas fluorescens, an organism not usually thought of as being oligotrophic, in carbon-free media is directly proportional to the permeability of the culture vessel closure. Conrad & Seiler (1980; 1982) have demonstrated a similar phenomenon in the utilisation of nanomolar concentrations of carbon monoxide by microorganisms in soil, sea water and fresh water. It is therefore clear that both in situ and in laboratory experiments the ability of bacteria to utilise miniscule quantities of atmospherically-derived carbon sources is important in both survival and competitiveness.

1.2) NUTRITIONAL PROPERTIES OF LOW NUTRIENT ENVIRONMENTS

1.2.1) Fresh water

The range of fresh water low nutrient environments includes pristine fast flowing streams, estuaries, lakes and a range of man-made environments. The levels of organic carbon in the lacustrine environment, the most studied oligotrophic fresh water environment, ranges from 1-26mg organic carbon l^{-1} . The traditional subdivision of lakes into oligotrophic and eutrophic types results in a "typical" oligotrophic lake being that with a photosynthetic primary production level and a total organic carbon flux of less than 0.1mg organic carbon $l^{-1} day^{-1}$. The equivalent values cited for the eutrophic system are between 3 and 5mg organic carbon $l^{-1} day^{-1}$ (Hood, 1970; Kuznetsov et al., 1979). As with other limits of this type, such subdivisions ignore such factors as the limited physical extent of the photic zone, the nature and localisation of other influxes of nutrients, the existence of interfaces, the potential effect of stratification on mixing of nutrients and, most importantly of all, the actual identity of the limiting factor itself and if it is a macronutrient whether it is carbon, phosphate or nitrogen.

Even in a carbon-limited natural ecosystem, simple measurements of total organic carbon concentrations tell only part of the story. The differential roles of particulate organic carbon (POC), typically highly resistant to bacterial

attack, and the more readily available dissolved organic carbon (DOC) are frequently ignored in considerations of oligotrophic environments (e.g. Kuznetsov et al., 1979; Poindexter, 1981a). The levels of readily assimilable DOC components, such as amino-acids and sugars, are extremely low. Jorgensen & Sondergaard (1984) have demonstrated that although amino-acids in the DOC fraction of an oligotrophic lake are readily available in solution they represent less than 0.2% of that fraction. Similarly sugar levels, typically 5µg sucrose l⁻¹ and 2µg glucose l⁻¹, (Kuznetsov et al., 1979) represent an extremely low proportion of the DOC fraction. In a detailed analysis of DOC composition in a woodland stream, Bott et al. (1984) obtained values of 0.7% peptides plus amino-acids, 7.5% monosaccharides, 20.1% total carbohydrates and 8.1% phenolics, i.e. they could identify less than 30% of the fraction. Thus even the apparently more accessible DOC fraction in the oligotrophic ecosystem will not readily supply the quantities of organic carbon suggested by simple analytical procedures and hence the nutrient limitation on the bacteria therein is far more complex than the simplistic concept of oligotrophy would have us believe.

1.2.2) Sea water

The bulk volume of the oceanic environment, in comparison to the relative sizes of the photic primary production zone and other localised nutrient-rich zones, for example hydrothermal vent areas (Jannasch, 1984), makes this the most important low nutrient environment, although again the potential for

multi-nutrient limitation and the effects of temperature and pressure must be emphasised. Below the photic zone in the open ocean DOC levels are normally within the range $0.35\text{--}70\text{mg l}^{-1}$ and POC levels are $3\text{--}10\mu\text{g l}^{-1}$ (Menzel & Ryther, 1970). As with fresh water, such figures represent oversimplifications, the resistance of both DOC and POC to microbial attack is great. Indeed, Barber (1968) detected no significant utilisation of deep ocean DOC over a two month incubation period in vitro despite the presence of viable bacteria in the samples. Whilst it has been shown that hydrolysed deep ocean POC can serve as a bacterial growth substrate (Gordon, 1970), it appears that natural marine populations metabolise very slowly (Craig, 1971; Williams et al., 1969) and in vitro studies confirm this with net population generation times of between 11 and 210 hours determined (Carlucci & Williams, 1978; Jannasch, 1969). This type of observation has lead to the suggestion that the majority of bacterial activity in the marine ecosystem, in common with the other low nutrient environments, occurs at interfaces where there is local concentration of nutrients (Fletcher & Marshall, 1982; Wardell et al., 1983). Also of importance in the abyssal oceanic environment will be microbial associations with deep ocean animals (Deming et al., 1981; Deming & Colwell, 1982).

1.2.3) Soil

The heterogeneous nature of soil makes it unlikely that any widespread areas will be oligotrophic in nature. However, it

is as a consequence of this heterogeneity that microcosms within the habitat will individually be of differing trophic statuses. It has been calculated that even within comparatively rich deciduous woodland soils the mean population generation time is in excess of 20 hours (Gray, 1976) and within such an environment differing communities will be subjected to different degrees of stress. As with other oligotrophic ecosystems, the bulk of available organic carbon, excepting a proportion of that from plant roots and the microfauna, will be in the form of relatively resistant compounds, such as cellulose and lignin, and that other environmental factors, including temperature and water availability, will also play a part in the limitation of oligotrophic populations. The observations of Conrad & Seiler (1980; 1982) regarding the utilisation of trace levels of carbon monoxide by soil bacteria suggests that the nature of the oligotrophic microflora of soil is more complex than can be accounted for by simple measurements of soil nutrient levels.

1.3) THE BACTERIAL FLORA OF LOW NUTRIENT ENVIRONMENTS

The degree of coverage in this section must of necessity be highly selective and is certainly open to criticism with respect to the breadth of the survey. In particular, many studies involving isolation of the bacteria of oligotrophic environments used media that could not possibly yield a representative sample of the bacteria therein. During the

course of this discussion I shall not dwell on such matters and intend this section merely to serve as a general guide to the microflora of low nutrient environments and wish to emphasise that such limited surveys do not yield any data regarding bacterial activities in situ.

1.3.1) Fresh water

The natural fresh water oligotrophic environment most studied with regard to the microflora is the lacustrine ecosystem. Data on populations in oligotrophic stream and river ecosystems is lacking. In fast moving pristine rivers bacterial growth on large surfaces is severely restricted by physical stresses whilst surfaces less subject to turbulent flow, in particular suspended particle surfaces, will presumably play an important role in the persistence of the indigenous bacterial flora. However, detailed studies on the oligotrophic river environment are, presumably as a consequence of sampling difficulties, somewhat limited. The importance of biotic and abiotic surfaces and of primary photosynthetic production in the provision of nutrient sources has been noted (Geesey & Costerton, 1979; McFeters et al., 1978).

By its very nature, the lacustrine environment is more amenable to study and again surface growth is highly significant in terms of net biomass activity. For example, Kirchman & Mitchell (1982) demonstrated that although less than 10% of bacteria in pond and marsh ecosystems were surface-attached they accounted for in excess of 40% of

^{14}C -glutamate and ^{14}C -glucose utilisation in vitro.

The nutrient sources available to the organisms in situ are unlikely to be as simple. Bell (1984) has demonstrated that natural oligotrophic populations can utilise algal extracellular products as sole carbon sources. Ishida & Kadota (1981) have shown that natural oligotrophic populations grow excellently on glycollate, a common algal and cyanobacterial photosynthetic exudate. These compounds, in conjunction with nutrient input by means of streams and rain run-off, are likely to be the only significant carbon sources available to heterotrophs. Certainly, the populations observed are well adapted to an oligotrophic mode of life. Witzel et al. (1982) have shown that many isolates from Lake Plußsee are capable of oligocarbophilic growth and Currie & Kalff (1984) have shown that in chemostat culture an isolate of Pseudomonas paucimobilis can outcompete an alga, its sole source of carbon, for phosphate and for exogenously introduced carbon. In the light of such evidence the apparently simplistic observations of Ishida et al. (1980) and Bell & Albright (1982) that isolates of "oligotrophic" free-floating bacteria preferentially utilise amino-acids as carbon sources whereas "eutrophic" forms preferentially utilise carbohydrates appear to be irrelevant with regard to the natural environment.

Whilst in many lakes the bulk of the population consists of morphologically indistinct and, apparently, highly adapted cells, including forms identified as Pseudomonas, Vibrio, Staphylococcus, Flavobacterium

and various coryneform genera (Witzel et al., 1982), it is the morphologically more unusual cell types, commonly restricted to the oligotrophic environment, that have attracted most attention. Of particular interest are the prosthecate and non-prosthecate budding bacteria (see Section 1.4). These have been observed in low nutrient fresh water environments for many years (Henrici & Johnson, 1935) and their ubiquity in both natural populations and in enrichments frequently noted. I do not intend to provide an exhaustive list of the types and sources of these organisms, this having been extensively reviewed elsewhere (Nikitin & Kuznetsov, 1967; Hirsch & Rheinheimer, 1968; Hirsch & Pankratz, 1970; Morgan & Dow, 1985; 1986). In a survey of a number of lakes of differing trophic status, Dow & Lawrence (1980) observed that the frequency of prosthecate and budding bacteria in the population was inversely proportional to the nutrient concentration. It was also observed that during static batch enrichment in the laboratory the numbers of prosthecate and budding bacteria increased with time (Fig. 1.1) and that this response reflected the competitiveness of these organisms under conditions of increasing nutrient stress (Lawrence, 1978). The prosthecate bacteria are frequently found attached to surfaces in the natural environment and often to other cells, including cyanobacteria and algae (Gromov, 1964; Paerl, 1976), holotrichs (Fauré-Frémiet, 1950) and bacteria (Callerio et al., 1983).

The third type of natural fresh water environment I shall consider is the estuarine ecosystem. Mallory et al.

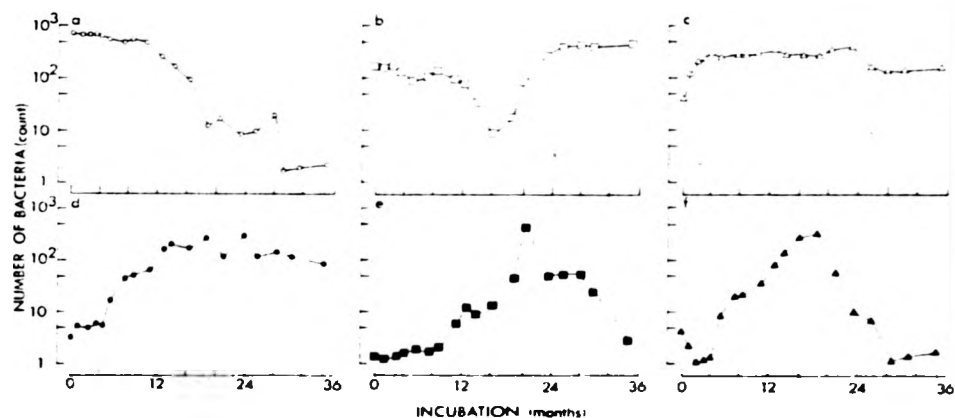


FIGURE 1.1 - Development of fresh water oligotrophic bacterial populations during enrichment culture. Data presented are of relative numbers of morphological forms of bacteria developing during dark, static, room temperature incubation of water taken from Draycote Reservoir, Warwickshire. The values given were obtained by direct cell-type counts of approximately 1000 cells under the electron microscope. (a) rods, cocci and spirilla. (b) Hyphomicrobium and related forms. (c) Caulobacter and related forms. (d) multiprosthate cells. (e) gas vacuolated rods. (f) Planctomyces. Data from Lawrence (1978). Figure from Morgan & Dow (1985).

(1977) observed a range of organisms including morphologically indistinct organisms as well as prosthecae and budding bacteria in low nutrient areas of estuarine environments. MacDonnell & Hood (1982) did not observe prosthecae bacteria in their samples but noticed that many of the organisms were extremely small and could pass through 0.2µm membrane filters, a situation common in the marine environment (see below). In both cases a range of organisms were isolated, representing both morphologically "typical" genera, for example Pseudomonas, Vibrio, Alcaligenes, Planococcus and Corynebacterium, and morphologically more distinct forms, for example Sphaerotilus, Hyphomicrobium and Hyphomonas.

There also exist a number of man-made low nutrient environments. Sly & Hargreaves (1984) isolated a range of bacteria capable of growth in swimming pool water, including a new species of budding bacteria. The environment that has received most study is distilled water. It has been noted that a range of potentially pathogenic enteric bacteria and Pseudomonas aeruginosa are capable of growth in tap and distilled water (Botzenhart & Kufferath, 1976; Favero et al., 1971; van der Kooij et al., 1980; 1982). Of the apparently more specialised oligotrophic bacteria, prosthecae bacteria are commonly observed in distilled water, often as contaminants of electron microscope preparations (Houwink, 1952; Callerio et al., 1983).

1.3.2) Sea water

Zobell & Upham (1944) stated that "besides the specific salinity requirements of marine bacteria, they are not morphologically or physiologically distinctive" and studies on the bacterial flora of the sea water environment have yielded a range of organisms analogous to that found in fresh water oligotrophic environments. In the bulk phase of the environment a range of forms can be isolated, including the morphologically distinct genera Planctomyces (Bauld & Staley, 1976), Caulobacter (Jannasch & Jones, 1960) and Hyphomicrobium (Hirsch & Rheinheimer, 1968). Attempts have been made to classify bacterial populations in the bulk phase into oligotrophic and copiotrophic groupings by means of their responses to nutrient levels in laboratory culture. As is the case with fresh water, such studies yield data of questionable value. Yanagita et al. (1978), Akagi et al. (1977; 1980) and Horowitz et al. (1983) all reported that groupings of the two trophic forms of bacteria existed in sea water and that representative taxa in both populations were similar and included Pseudomonas and Vibrio species.

The activity of organisms at interfaces in the oceanic environment has also received attention. Zobell & Upham (1944) stated that many forms were attached in the natural environment and the importance of such processes has since received much attention (Fletcher & Marshall, 1982). In a study of the taxa involved in colonisation of wood surfaces in a tropical harbour (Austin et al., 1979) a range of dominant genera were isolated which included Vibrio.

Pseudomonas, Bacillus and the prosthecate genera Hyphomicrobium and Hyphomonas. A complex pattern of community development and succession was described with old surfaces supporting a highly heterogeneous flora. The patterns of community development at interfaces in the natural low nutrient environment have attracted a good deal of interest in recent years (Fletcher & Marshall, 1982) but detailed information on the organisms and interactions therein is still lacking. In the light of present evidence the importance of interface populations in the oligotrophic sea water environment would appear to be great.

The feature of bacteria in the oligotrophic oceanic environment that has attracted the greatest degree of attention is the fact that many of the cells are extremely small. These ultramicrobacteria (Torella & Morita, 1981), the properties and production of which will be considered below (Section 1.5.4), are characterised as that proportion of the population which will pass through 0.45µm membrane filters (Tabor et al., 1981), although many will also pass through 0.2µm filters (Morita, 1982). It appears that the majority of marine bacteria, including Pseudomonas, Flavobacterium and Vibrio species, are capable of producing ultramicrocells (Tabor et al., 1981) and that such forms represent the dominant proportion of the flora under normal conditions. Such is their ubiquity and physiological nature (Section 1.5.4) that many workers believe them to be the true native bacteria of the oligotrophic open ocean (Novitsky & Morita, 1977; Stevenson, 1978; Jones & Rhodes-Roberts, 1981).

1.3.3) Soil

The physical structure of soil makes study of bacterial populations difficult without major disruption to the structure of the environment. As a consequence, a number of investigations, rather than using crude cell extraction techniques, have used methods that attempt to maintain the physical discontinuity and structure of the environment. Most important of these techniques in the study of oligotrophic microsites has been the use of microcapillary tubes which are designed to penetrate and extend a microcosm and enable direct observations on the populations therein (Perfilev & Gabe, 1969). By means of such techniques detailed observations on the bacteria in soil have been made and the wide morphological and physiological variety of organisms within given niches noted (Aristovskaya, 1963; Perfilev & Gabe, 1969). In detailed electron microscopic studies of oligotrophic soil bacteria, Aristovskaya (1963) and Nikitin et al. (1966) observed a number of distinct cell-types including the budding bacterium Seliberia and a number of prosthecate, multi-prosthecate, sheathed and stellate cells. As is the case with aquatic oligotrophic environments, prosthecate bacteria are commonly observed and these are frequently attached to abiotic and biotic surfaces, the latter including diatoms (Nemec & Bystricky, 1962) and rhizosheaths (Wullstein & Pratt, 1981), these presumably being local sources of nutrients.

The nature of the morphologically "typical"

oligotrophic bacteria in soil is less clear. Hattori (1981; Hattori & Hattori, 1980) cultivated soil populations on agar plates of various dilutions of nutrient broth and observed that many forms were inhibited by "high" concentrations of nutrients and proposed an "index" of oligotrophy based on the proportion of the population that does not grow on nutrient-rich medium. Such a suggestion again ignores questions of medium choice, in situ physical conditions and community structure and does not appear to have gained widespread acceptance. In the light of the observations of Conrad & Seiler (1980; 1982) that non-culturable bacteria in soil can utilise extremely low concentrations of carbon monoxide as a carbon source, the probability that similar bacteria exist which are capable of utilising higher molecular weight carbon sources at similar levels, cannot be ignored.

1.3.4) Conclusions

Up to now I have solely considered observations on the physical and microbiological nature of the oligotrophic environments. The arbitrary definitions of oligotrophy chosen by many workers and the isolation techniques employed are, in the light of observations on the natural environment, somewhat naive, relying as they do on the use of dilutions of carbon-limited media, selective cultivation of the population and arbitrary dividing lines. Much of the available data concerning the bacterial flora of low nutrient ecosystems ignores the heterogeneity of the environments and does not

consider the activity of the organisms in situ. The ubiquity of extreme low nutrient environments and their importance on the global scale makes it likely that there are a range of adaptations in bacteria for a successful existence in the oligotrophic ecosystem. Particularly common in such environments are the prosthecate and budding bacteria and in the remainder of this introduction I shall consider these organisms in detail and the adaptations they and other bacteria may possess for growth in low nutrient environments.

1.4) THE PROSTHECATE AND BUDDING BACTERIA

1.4.1) Introduction

Budding or, more correctly, unidirectional polar growth, is a pattern of bacterial growth that is a consequence of the insertion of new cell material at one single active growth point (Kelly & Dow, 1984). Whilst the more typical laboratory organisms grow by means of several active growth points in nutrient-rich media, Escherichia coli when growing under conditions of nutrient stress with a doubling time of in excess of 60 minutes appears to grow polarly (Donachie & Begg, 1970; Begg & Donachie, 1973; 1977). In this section I intend to consider a selection of those bacteria with an obligate budding mode of growth.

The production of new cell envelope material at a single site may reflect the inability of the cell to support multiple points under conditions of nutrient limitation and in addition may have a number of potential consequences and

roles. The production of new material at one pole of metal encrusted attached cells, for example Pedomicrobium, may be a mechanism for the production of new cells away from the encrustation and hence be an aid to dispersal (Aristovskaya, 1963). More generally, as a function of asymmetric insertion of new material into the envelope, the two cells produced at division, which itself may (or may not) be asymmetric, can be distinct. If one progeny cell consists largely or entirely of new cell material as a consequence of polar growth it will be functionally younger than the other and as a result there may be the production of two distinct cell types, one of which is immature and the second of which is potentially aged (Whittenbury & Dow, 1977). There is therefore the possibility of functionally distinct cell type expression (Moore & Hirsch, 1973a; Kelly & Dow, 1984). Furthermore, if the growth point is polar then the cell itself must be polar and possess distinct front and back ends. This concept and the potential for cell type expression are clearly demonstrated by Rhodopseudomonas palustris (Fig. 1.2; Kelly & Dow, 1984). In this organism photosynthetic membrane is not distributed to the daughter cell. Consequently, this cell can respond to a changed light regime during de novo photosynthetic membrane synthesis and hence represents an immature cell type. The polarity of the daughter cell is maintained - when the flagellum is shed the flagellated pole becomes the site of holdfast synthesis and the pole at which cell division occurred becomes the polar growth point.

Prosthecae are extensions of the bacterial cell that

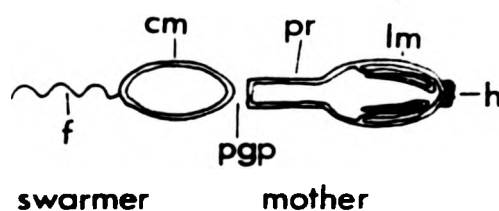


FIGURE 1.2 - Diagrammatic representation of asymmetry in *Rhodospseudomonas palustris*. The two cell types (mother cell and daughter or swarmer cell) are oriented with respect to internal and external structures and growth points. The division pole gives rise to the growing point and holdfast development is at the flagellated pole. Furthermore, each cell is morphologically and physiologically distinct, for example the swarmer cell contains none of the parental lamellate membrane system. f - flagellum; cm - cytoplasmic membrane beneath cell wall; pgp - polar growth point; pr - prostheca; lm - lamellate membrane system beneath cell wall; h - holdfast. After Kelly & Dow (1984).

TABLE 1.2 - Described genera of prosthecate bacteria.

<u>Genus</u>	<u>Comments</u>	<u>Selected references</u>
<u>Caulobacter</u>		Poindexter, 1981b.
<u>Asticcacaulis</u>		Pate <u>et al.</u> , 1973.
<u>Hyphomicrobium</u>	Facultative methylo-troph.	Harder & Attwood, 1978.
<u>Hyphomonas</u>		Moore <u>et al.</u> , 1984.
<u>Rhodomicrobium</u>	Photoheterotroph, Rhodospirillaceae.	Whittenbury & Dow, 1977.
<u>Prosthecochloris</u>	Phototroph, Chlorobiaceae	Gorlenko, 1970
<u>Ancalochloris</u>	Phototroph, Chlorobiaceae.	Gorlenko & Lebedeva, 1971; Trüper & Pfennig, 1981.
<u>Stella</u>	Star-shaped.	Vasileva, 1970; Vasileva <u>et al.</u> , 1974.
<u>Angulomicrobium</u>	"Mushroom" or Triangular shape.	Whittenbury & Nicoll, 1971; Lafitskaya & Vasileva, 1976; Vasileva <u>et al.</u> , 1979.
<u>Prosthecomicrobium</u>	May be multiply appendaged.	Vasileva <u>et al.</u> , 1974; Staley, 1968.
<u>Ancalomicrobium</u>	May be multiply appendaged	Staley, 1968; Lawrence, 1978.

<u>Labrys</u>		Vasileva & Semenov, 1984.
<u>Prosthecobacter</u>		de Bont <u>et al.</u> , 1970; Staley <u>et al.</u> , 1976.
<u>Thiodendron</u>	Deposits sulphide.	Perfilev, 1969; Schmidt, 1981.
<u>Pedomicrobium</u>	Deposits heavy metals.	Gebbers & Hirsch, 1978.
<u>Metallogenium</u>	Manganese oxidiser.	Zavarzin, 1981.

are cellular in nature and continuous with the main body of the cell itself (Staley, 1968). The variety of morphological and physiological types of prosthecae bacteria (Table 1.2), as well as taxonomic studies (see below), suggest that such structures have evolved independently several times. The terms stalk or fascicle are applied to prostheca-like acellular structures which are also produced by organisms commonly observed in oligotrophic environments, e.g.

Blastocaulis, Planctomyces (Tekniepe et al., 1981; Schmidt & Starr, 1978; 1981; 1982), and forms related to Gallionella (Hanert, 1981).

Many roles for prosthecae have been proposed. In certain forms, for example Hyphomicrobium and Rhodomicrobium, they play a role in reproduction, daughter cell formation occurring at the distal end of the prostheca.

In such cases the prostheca may serve to extend the physical separation produced by polar growth and facilitate separation of DNA (Hyphomicrobium; Moore & Hirsch, 1973b), photosynthetic membrane (Rhodomicrobium; Whittenbury & Dow, 1977) or other cellular components. They may be involved in suspension, helping maintain cells at the air-water interface (Poindexter, 1964). Indeed, some strains of the prosthecate genera Prosthecomicrobium and Ancalomicrobium possess gas vacuoles to aid this process (Staley, 1968; Walsby, 1976). Many prosthecate bacteria attach to interfaces by means of holdfasts at the distal ends of the prosthecae, although not all holdfast-producing prosthecate bacteria involve their prosthecae in this way (e.g. Asticcacaulis; Pate *et al.*, 1973). Moreover, at one time the attachment of prosthecate bacteria to other cells in the natural environment via the holdfast at the end of the prostheca was believed to represent parasitism by these organisms with the prostheca acting as a sucking proboscis (Houwink, 1955). The property most commonly attributed to prostheca is that they function as mechanisms for increasing the surface area to volume ratio of the cell. In the phototroph Prosthecochloris, photosynthetic membrane within the prosthecae (Gorlenko, 1970) suggests that they function to increase the surface area available for photosynthesis. Many prosthecate bacteria increase prostheca length in response to decreasing nutrient levels (Schmidt & Stanier, 1966) and in Ancalomicrobium (Lawrence, 1978) a range of morphological cell types are expressed under differing nutrient

concentrations with surface area increasing with increasing nutrient stress (Fig. 1.3).

As described above (Section 1.3) the most fruitful environment for finding prosthecate bacteria is the oligotrophic one, an observation that has led to the suggestion that prosthecate bacteria are model oligotrophs (Poindexter 1981a; 1981b). Whilst the ubiquity of prosthecate bacteria in such environments is clear, it is important to note that prosthecate bacteria are also observed in significant, though proportionally lower, numbers in certain eutrophic aquatic environments (Dow & Lawrence, 1980; Poindexter, 1981b). Furthermore, certain specific nutrient-rich environments contain very high populations of prosthecate bacteria. Such described environments include a eutrophic solar lake (Hirsch, 1977), a cutting fluid sludge reactor (Baker et al., 1983), alkane polluted oceanic waters (Murakami et al., 1976), wood pulp waste lagoons (Stanley et al., 1979) and pus recovered from a case of human sinusitis (Pongratz, 1957). In the latter context it is important to note that this isolate and all other prosthecate bacteria tested are not pathogenic (Famurewa et al., 1983).

1.4.2) Caulobacter and Asticcacaulis

The genus Caulobacter was first proposed by Henrici & Johnson (1935) to incorporate the "stalked" bacteria they observed attached to microscope slides immersed in a fresh water lake. As presently defined (Poindexter, 1964), the genus consists of truly prosthecate aerobic chemohetero-

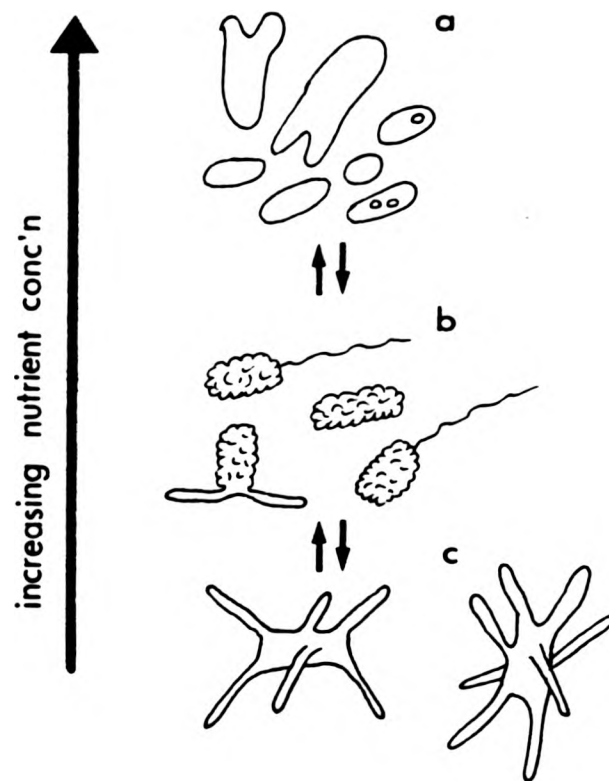


FIGURE 1.3 - Effects of nutrient concentration on the morphology of Ancaelomicrobium. Three interchangeable morphological forms can be recognised whose expression is dependent on environmental nutrient concentration. (a) morphologically simple form produced at "high" nutrient concentrations. (b) Characteristic lobate form produced at intermediate nutrient concentrations. (c) multiprosthete form produced at very low nutrient conditions. Data from Lawrence (1978).

trophic bacteria which may possess a polysaccharide holdfast at the distal end of the prostheca and reproduce by budding at the non-prosthecate pole of the cell. Two distinct cell types are consequently produced at division, the prosthecate mother cell and the polarly flagellated daughter (swarmer) cell, and a complex dimorphic cell cycle exists (Fig. 1.4). The period of development of the latter cell type is temporally variable and is apparently under environmental control - development does not occur until environmental conditions are suitable for reproduction to occur (Dow et al., 1983). Following the onset of development, a sequence of obligate stages of differentiation occurs (Fig. 1.4) resulting in the production of two cell types on division. The prosthecate cell can immediately undergo a second round of reproduction provided environmental conditions are conducive (mother cell cycle) whereas the development of the swarmer cell is again under environmental control. In laboratory culture variations in nutrient conditions alter the relative proportions of the two cell types during growth (Poindexter, 1984a) but the dimorphic cycle is obligate. At least 19 species of Caulobacter are recognised (Poindexter, 1981b), although the differentiation between species relies heavily on considerations of morphology and pigmentation. All of these species possess similar dimorphic life cycles but physiological studies to date have concentrated on two species, C. crescentus and C. vibrioides, which are regarded as being atypical in that they exhibit more rapid growth and a greater degree of nutritional independence than

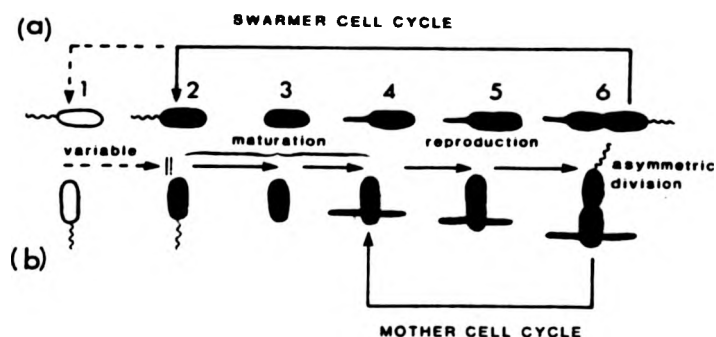


FIGURE 1.4 - Cell cycles of (a) *Caulobacter* and (b) *Asticcacaulis biprosthecum*. Both cycles are functionally identical. Stage 1 represents the swarmer cell which will not undergo differentiation and reproduction until environmental conditions are suitable. Following initiation of differentiation, a sequence of obligate morphological stages occur, starting with the loss of the polar flagellum (stages 2 and 3) followed by prostheca synthesis (stage 4). Growth of the cell body (stage 5) is followed by synthesis of a flagellum at the distal end of the daughter cell (stage 6) and asymmetric cell division to yield two cell types. The prosthecate mother cell can immediately undergo a further round of reproduction whereas development of the swarmer cell is again under environmental control. As a consequence two distinct cell cycles can be identified: that of the mother (prosthecate) cell and that of the swarmer cell which includes a temporally variable period. Figure from Morgan & Dow (1985).

the majority of described forms (Poindexter, 1981b).

The genus Asticcacaulis (Poindexter, 1964) contains organisms that are physiologically analogous to Caulobacter and possess a functionally identical life cycle (Fig. 1.4) but which produce prosthecae excentrally and the polysaccharide holdfast on the main body of the cell (Umbreit & Pate, 1978). Two species are recognised: A. excentricus (Poindexter, 1964) and A. biprosthecum (Pate et al., 1973).

The cell cycle of Caulobacter has received widespread attention as a model prokaryotic differentiation system. I do not intend to review in detail the large amount of work performed because the bulk of the topic is somewhat outside the scope of this report and has received excellent coverage elsewhere (e.g. Wood & Shapiro, 1975; Shapiro, 1976; Poindexter, 1981b; Dow et al., 1983). Developing populations of Caulobacter swarmer cells have been investigated in detail from a number of angles and a variety of significant physiological differences between the two cell types noted. In the swarmer cell the overall rate of protein synthesis is significantly lower than that occurring in the prosthecae cell and is apparently controlled at the transcriptional level (Iba et al., 1978). More specifically, the expression of a number of proteins is restricted to the individual cell types and to temporally definable stages during the obligate differentiation pathway (Milhausen & Agabian, 1981). Furthermore, the polarity of the reproducing cell not only includes the production of the

flagellum at the pole of the developing swarmer cell but also a number of other membrane-associated and soluble proteins (Agabian et al., 1979; Huguenal & Newton, 1984). Also specific to swarmer cells are methyltransferases involved in chemotaxis (Shaw et al., 1983). At the DNA level, the nucleoids of C. crescentus CB15 swarmer cells have different sedimentation properties associated with a more compact structural organisation and a consequently lower level of transcription (Swoboda et al., 1982a; 1982b). DNA synthesis does not occur in the swarmer cell and only commences during differentiation concomitant with prostheca synthesis (Degnen & Newton, 1972; Iba et al., 1977) or the equivalent period in prostheca-deficient mutants (Fukuda et al., 1977). This range of evidence all points to the swarmer cell being a metabolically quiescent cell type (Dow et al., 1983).

Both Caulobacter and Asticcacaulis are aerobic chemoheterotrophs capable of utilising a range of sugars, amino-acids and organic acids as carbon sources. Whilst the physiology of the organisms has received comparatively little attention, it has been shown that Caulobacter possesses a full tricarboxylic acid (TCA) cycle in all cell types (Riley & Kolodziej, 1976) and utilises the Entner-Doudoroff pathway to metabolise carbohydrates (Riley & Kolodziej, 1976; Kurn et al., 1978). Specific enzymes of the pathway and the activity of glucose-6-phosphate dehydrogenase are inducible (Shedlarski, 1974; Kurn et al., 1978). Chemotaxis of C. vibrioides swarmer cells to a range of amino-acids has

also been demonstrated, with glycine being a repellent (Matveyeva & Gromov, 1983). The organism can also accumulate poly- β -hydroxybutyrate (PHB) and polyphosphate (PP.) storage compounds (Poindexter & Eley, 1983).

The aspect of the physiology of Caulobacter that has received the most attention is the potential role of the prostheca. The presence of cross-bands in prostheca has frequently been noted and are of unknown function but they do not (Swoboda & Dow, 1979) represent markers of cell division as was proposed by Staley & Jordan (1973). Observations that prostheca length increases under conditions of increasing nutrient stress, and particularly phosphate limitation (Schmidt & Stanier, 1966), has lead to the suggestion that Caulobacter prosthecae function not only generally for increasing the surface area to volume ratio but are specialised sites for the uptake of inorganic phosphate (Poindexter, 1984a; 1984b). Evidence against this proposition appears great. A number of workers have observed prostheca elongation in response to carbon and nitrogen limitation alone (e.g. Hirsch & Rheinheimer, 1968; Poindexter, 1979) and Larson & Pate (1976) have demonstrated the existence of high and low affinity glucose transport systems in isolated prosthecae of Asticcacaulis biprosthecum.

The nature of the physiology of these organisms has lead to the suggestion that they represent model oligotrophs (Poindexter, 1981a; 1981b). In particular, Poindexter (1981b) believes that Caulobacter has low upper limits of nutrients above which growth is inhibited. In contrast, Nikitin

et al. (1982) have shown that C. vibrioides is unlike the proposed model oligotrophs in that it has a requirement for comparatively high concentrations of growth factors and a limited range of carbon substrates. The observation and isolation of Caulobacter-like organisms, frequently possessing very long prosthecae, in oligotrophic soil, fresh water and sea water environments is a commonly reported event (for reviews see Poindexter, 1981b; Morgan & Dow, 1985), although observation of Asticcacaulis is rare (Poindexter, 1981b). However, Caulobacter has also been observed in a number of comparatively nutrient-rich environments (e.g. Staley, 1971; Stanley et al., 1979). It is probable that Caulobacter is adapted to and highly successful in the oligotrophic environment and that in the natural ecosystem it will normally be attached to surfaces (Poindexter, 1964; 1981b). However, it also appears to be successful in specific eutrophic environments and is probably ubiquitously distributed in nature.

1.4.3) Hyphomicrobium and related forms

These are chemotrophic prosthecate bacteria which reproduce by means of bud formation at the ends of their prosthecae. The distinctive morphology of these organisms resulted in their observation as long ago as the end of the nineteenth century (Henrici & Johnson, 1935; Harder & Attwood, 1978). There are presently three recognised morphologically similar genera, Hyphomicrobium, Hyphomonas and Pedomicrobium.

An increasing amount of taxonomic work has been

performed on these organisms and serological (Powell et al., 1980) and DNA-DNA hybridisation studies (Moore & Hirsch, 1972) suggest that differentiation between Hyphomonas and Hyphomicrobium is valid. The morphology of these two genera is identical and physiological differentiation between the two is based on the fact that Hyphomicrobium is a restricted facultative methylotroph (Harder & Attwood, 1978), whereas Hyphomonas can utilise more complex compounds as carbon sources (Moore et al., 1984). The taxonomic status of Hyphomicrobium is so unclear that Harder & Attwood (1978) were of the opinion that details of only one species, H. vulgare, have been validly published. As a consequence, most isolates of the organism are referred to by means of identifying characters alone and not by specific epithets. The organism is capable of growth on one-carbon compounds and a very few two and four-carbon compounds (ethanol, acetate and 3-hydroxybutyrate; Harder & Attwood, 1978). The genus Hyphomonas contains five species, including H. polymorpha, isolated from pus from a case of human sinusitis (Pongratz, 1957) and four marine species (Moore et al., 1984; Weiner et al., 1985). The genus Pedomicrobium is differentiated from Hyphomicrobium and Hyphomonas by means of its ability to precipitate metal hydroxides on the cell surface, the production of up to five prosthecae which may be away from the poles of the cell and the utilisation of a wide variety of carbon sources (Moore, 1981; Moore et al., 1984). Three species are recognised which are arbitrarily divided on the basis of which metal

hydroxides are precipitated during growth (Moore, 1981): P. ferrugineum precipitates iron salts; P. manganicum manganese salts; P. podsolicum precipitates both iron and manganese salts.

The cell cycle of Hyphomicrobium (Moore & Hirsch, 1973a; Fig. 1.5) involves the production of two cell types, swarmer and mother cells, analogous to those observed in Caulobacter. In normal laboratory culture a simplified life cycle is observed with prosthecae cells producing polarly flagellated swarmer cells at the end of the prosthecae which are released by asymmetric division. As is the case with Caulobacter, the mother cell can immediately undergo a second round of reproduction whereas development of the swarmer cell is under environmental control. In the natural environment and in culture under extreme nutrient stress, Hyphomicrobium is normally observed as chains of cells (Dow et al., 1983; Fig. 1.5) with occasional swarmer cell production enabling dispersal. It is this growth mode that is probably the norm for Hyphomicrobium in the natural environment. Morphological variation in the main cell body of Hyphomicrobium grown under different conditions has been reported (Lawrence, 1978; Harder & Attwood, 1978). In common with Caulobacter, a number of physiological differences between prosthecae and swarmer cells have been reported for Hyphomicrobium. Differential protein expression at different stages of the cell cycle has been demonstrated (Matzen, Dow & Hirsch, unpublished observations). DNA synthesis only occurs once prosthecae synthesis has begun

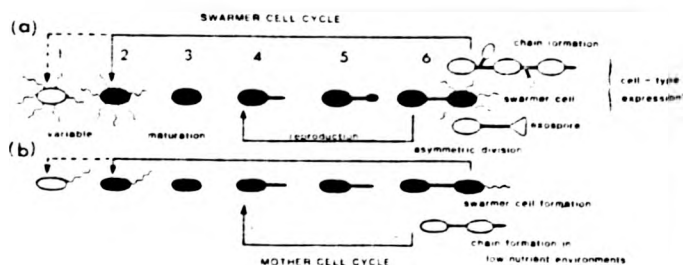
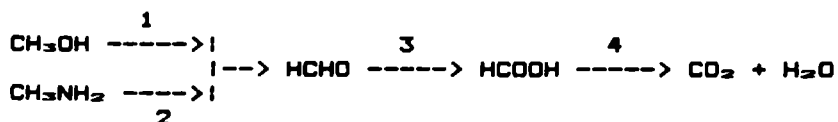


FIGURE 1.5 - Cell cycles of (a) Rhodospirillum rubrum and (b) Rhodospirillum rubrum. There is a fundamental similarity between the two organisms in that morphological patterns are dependent on environmental conditions. Development of the swarmer cell (stage 1) is under environmental control and once differentiation has begun an obligate sequence of morphological stages occurs proceeding via the loss of flagella (stage 2) and prostheca synthesis (stage 3) to bud growth (stages 4 & 5). At this point the cell cycle can proceed in a number of ways dependent upon environmental conditions. In comparatively high nutrient conditions (low light levels and high CO₂ concentrations for Rhodospirillum rubrum) a simplified cell cycle occurs with swarmer cell production and distinct mother and swarmer cell cycles being discernable. Under more limiting nutrient conditions, such as those observed in the natural environment, both organisms tend to suppress swarmer cell formation and produce chains of cells with occasional swarmer cell formation enabling dispersal. Under very strict nutrient limitation Rhodospirillum rubrum can produce triangular exospores. Figure from Morgan & Dow (1985).

(Wali et al., 1980) and on completion of synthesis the daughter cell nucleoid is translocated along the prostheca into the developing daughter cell (Moore & Hirsch, 1973b). The general physiological activity of the swarmer cell is reduced with low levels of RNA and protein synthesis (Wali et al., 1980) and a reduced adenylate charge (Emala & Weiner, 1983).

The physiology of Hyphomicrobium has been extensively studied (reviewed by Harder & Attwood, 1978). Hyphomicrobium is capable of aerobic chemoheterotrophic metabolism and can also grow under anaerobic conditions using nitrate as a terminal electron acceptor (Attwood & Harder, 1972). Studies on energy-yielding metabolism of one-carbon compounds by radiolabelling (Uebayasi et al., 1984) and enzymological techniques (Harder & Attwood, 1975) have demonstrated the operation of the following pathway:



The enzymes involved are: 1) methanol dehydrogenase; 2) methylamine dehydrogenase; 3) formaldehyde dehydrogenase; 4) formate dehydrogenase. Reducing equivalents are produced at each step and are converted to energy via the respiratory chain (Harder & Attwood, 1978). Assimilation of these substrates is by a modified serine pathway (Harder et al., 1973; Attwood & Harder, 1978) which lacks isocitrate lyase (Attwood & Harder, 1977). Utilisation of two and four-carbon

compounds is via acetyl-CoA (Harder & Attwood, 1978). The restricted range of carbon sources utilised by Hyphomicrobium has aroused a great deal of interest. It was proposed (Harder et al., 1975) that this behaviour was a result of the inability of this organism to synthesise an active pyruvate dehydrogenase complex which, since pyruvate is a key intermediate in the metabolism of most compounds containing three or more carbon atoms, would account for the observed responses. This was elegantly confirmed by Dijkhuizen et al., (1984) who cloned genes of the pyruvate dehydrogenase complex from E. coli into Hyphomicrobium X and were then able to cultivate the latter organism using pyruvate and succinate as sole carbon sources.

As described above for Caulobacter, prosthecae of Hyphomicrobium elongate under conditions of nutrient limitation. Again this led Poindexter (1984a) to suggest that prosthecae function as specialised phosphate uptake sites in this organism but independent observations that both nitrogen and carbon limitation have an equally marked effect on prostheca length in Hyphomicrobium (Harder & Attwood, 1978; Hirsch & Rheinheimer, 1968) must cast doubt on this suggestion.

The ecology of these organisms is comparatively unclear. Hyphomicrobium-like forms are common in all types of low nutrient environment (Hirsch & Conti, 1964; Hirsch & Rheinheimer, 1968; Harder & Attwood, 1978), as is the biological production of one-carbon compounds (Harder & Attwood, 1978). Hyphomicrobium can readily be isolated from

oligotrophic environments by anaerobic incubation of samples with the addition of nitrate and methanol as the sole carbon source (Sperl & Hoare, 1971; Attwood & Harder, 1972). It is probable that under most conditions they exist as chains of cells attached to surfaces (Austin et al., 1979; Moore & Marshall, 1981; Dow et al., 1983). The ecology of Pedomicrobium appears to be similar with metal-encrusted communities occurring at surfaces in iron and manganese-rich water and soil (Ghiorse & Hirsch, 1979; Moore, 1981).

1.4.4) Rhodomicrobium

The genus Rhodomicrobium contains one species, R. vannielii (Duchow & Douglas, 1949), and is a purple non-sulphur bacterium with a primarily photoheterotrophic mode of growth. The cell cycle of R. vannielii (Fig. 1.5; Whittenbury & Dow, 1977) is fundamentally similar to, although more complex than, that of Hyphomicrobium. Yet again, differentiation of the swarmer cell is under environmental control and can be prevented in laboratory culture by maintaining cells in darkness. Once differentiation has been initiated an obligate developmental sequence is followed resulting in bud production, although the subsequent pattern of bud development is under environmental control (Fig. 1.5). In normal laboratory culture, and apparently under most natural environmental conditions, chain formation is favoured with occasional swarmer cell production enabling dispersal. If the organism is cultivated under conditions of low light levels and high CO₂ concentrations a simplified dimorphic cell

cycle occurs with continuous swarmer cell production from prosthecae cells. Under conditions of extreme nutrient stress Rhodomicrobium produces triangular exospores which exhibit a greater resistance than vegetative cells to high temperature, desiccation and ultraviolet irradiation (Gorlenko et al., 1974; Whittenbury & Dow, 1977).

The development of R. vannielii swarmer cells has been studied in detail as a model differentiation system (reviewed by Whittenbury & Dow, 1977; Dow et al., 1983). Differential protein synthesis and cell type-specific proteins have been demonstrated during the cell cycle (Dow et al., 1983; 1985) and a very low level of RNA synthesis occurs in swarmer cells (Dow et al., 1983). Evidence to suggest a difference in DNA-dependent RNA polymerase structure in prosthecae and swarmer cells also exists (Dow et al., 1983). Nucleic acid synthesis only commences when prosthecae synthesis is initiated (Potts & Dow, 1979) and the nucleoid of the swarmer cell is in a more condensed state and is transcriptionally more quiescent than that of the prosthecae cell (Dow et al., 1983). This range of evidence demonstrating a restricted metabolic activity in the swarmer cell of R. vannielii and the accumulation of undifferentiated swarmer cells under conditions unsuitable for growth has led to the suggestion that this cell type functions as a "shut down" dispersal cell in this organism and other prosthecae bacteria (Dow et al., 1983).

The general physiology of Rhodomicrobium has received little attention. Under normal growth conditions

Rhodomicrobium is an anaerobic photoheterotroph capable of using a variety of organic compounds as electron donors, carbon sources and terminal electron acceptors (Whittenbury & Dow, 1977) and is somewhat difficult to cultivate under other conditions. There is only a little information available concerning the intermediary metabolism of the organism (Whittenbury & Dow, 1977), although the ribulose-1,5-bisphosphate carboxylase oxygenase (RUBISCO) of R. vannielii has been studied and appears to be generally similar to that of some other purple non-sulphur bacteria (Taylor & Dow, 1980). The organism is capable of using organic or inorganic nitrogen sources and can fix nitrogen in the absence of such compounds (Potts, 1980). As with the other prosthecae bacteria already considered, nutrient depletion increases the length of the prosthecae (Whittenbury & Dow, 1977) and it is interesting to note that in laboratory culture R. vannielii is markedly inhibited by amino-acid concentrations of in excess of 0.01% (Duchow & Douglas, 1949).

The ecology of Rhodomicrobium is similarly unclear. The original isolation was from fresh water sediment (Duchow & Douglas, 1949) and the organism has subsequently been isolated from coastal, salt marsh, bulk fresh water and sulphur-rich waters (Hirsch & Rheinheimer, 1968; Nikitina et al., 1978). Little information is available regarding the occurrence of the organism under different trophic conditions, although it is probable that the organism normally exists as multicellular arrays in oligotrophic systems.

1.4.5) Planctomyces and related forms

The genus Planctomyces was believed for some 50 years to be a fungal genus (Starr & Schmidt, 1984) but actually consists of budding bacteria which possess acellular multifibrillar stalks with a holdfast at the distal end (Schmidt & Starr, 1978) and are discussed here because of the remarkable resemblance of their life cycles to those of the prosthecae bacteria considered above (Fig. 1.6). Hirsch (1972) first demonstrated that the original type species of the genus Planctomyces, P. bekefii, was, in fact, not fungal but bacterial and apparently identical to the form described by Henrici & Johnson (1935) as Blastocaulis sphaerica. The organisms of this type are presently classified in an arbitrary grouping as the Blastocaulis-Planctomyces group (Schmidt & Starr, 1981). Members of this group and the related non-stalked genus Pirella have peptidoglycan-free proteinaceous cell sacculi (Konig et al., 1984) and 16S rRNA studies (Stackebrandt et al., 1984) have suggested that they represent a distinct evolutionary line of prokaryotes which diversified from the archaeobacteria and eubacteria early in evolutionary history.

At present the taxonomy of the stalked budding bacteria is highly confused with a variety of organisms covered under the general heading of the Blastocaulis-Planctomyces group. The situation is further complicated by the lack of any isolate of the type species and the fact that most species are distinguished on

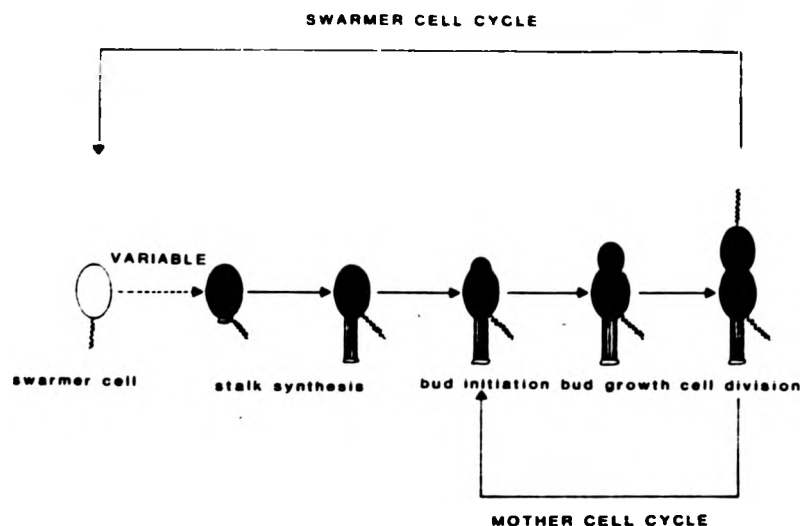


FIGURE 1.6 - Cell cycle of *Planctomyces*. The cycle is fundamentally identical to that of the prosthecate bacteria excepting the acellular nature of the stalk. Both mother and swarmer cell cycles can be recognised. After Tekniepe *et al.* (1981).

morphological grounds only and have never been isolated (Staley & Bauld, 1981). Schmidt & Starr (1978) classified Planctomyces into five morphological groupings for descriptive purposes and recently a number of species of Planctomyces have been validly described (e.g. Bauld & Staley, 1976; Starr et al., 1983; Starr & Schmidt, 1984).

The physiology of the organisms has not been studied, although it is known that they are aerobic chemoheterotrophs and are capable of storing PHB and PP_i (Schmidt & Starr, 1982). It is interesting to note that stalk formation in many isolates can be suppressed by growth in high nutrient medium (0.1% peptone + yeast extract as opposed to 0.001-0.01%; Schmidt & Starr, 1978).

Organisms resembling Planctomyces have been observed in a variety of oligotrophic fresh water environments, commonly in the presence of prosthecae bacteria (Henrici & Johnson, 1935; Lawrence, 1978; Schmidt, 1978) and in sea water (Bauld & Staley, 1976; Schmidt & Starr, 1981). They have also been observed in a number of eutrophic environments (Schmidt & Starr, 1981).

1.4.6) Other prosthecae bacteria

The genera Prosthecomicrobium and Ancalomicrobium were first described by Staley (1968) who observed and isolated them from oligotrophic fresh water. Both genera are multiprosthecae and are differentiated by their growth patterns, the latter genus growing by budding and the former by elongation at multiple growth points. A number of species

of Prosthecomicrobium have been proposed (Staley, 1981a; 1984; Bauld et al., 1983). However, the observation of Lawrence (1978; Fig. 1.3) that the morphology of these organisms is greatly altered under different nutrient conditions cautions against excessive reliance on morphological characteristics in the taxonomy of these organisms. Both genera are characteristic of the free-living microflora of oligotrophic environments and the production of longer and greater numbers of prosthecae under conditions of nutrient limitation suggests that they are highly adapted for this mode of existence.

The genus Prosthecobacter (Staley et al., 1976) is morphologically very similar to Caulobacter and is also found in oligotrophic environments. It has been little studied yet differs significantly from Caulobacter and related genera in that the daughter cell produced possesses a prostheca and is morphologically identical to the mother cell.

The genus Thiodendron was isolated from saline sulphur-rich waters (Perfilev, 1969) and consists of long thin vibrioid cells with prosthecae at one or both poles of the cell which occasionally are dichotomously branched. In nature they often produce thick multicellular arrays and deposit sulphur. A life cycle involving a coccoid swarmer cell has been described but has not been studied in detail.

The genera Labrys (Vasileva & Semenov, 1984) and Stella (Vasileva, 1970; Vasileva et al., 1974) are both radially symmetrical prosthecate bacteria. The latter genus,

as the name suggests, is stellate in morphology with (usually) six prosthecae arranged regularly in a radial pattern around a lenticular main cell body. The morphology of Labrys is similar but more irregular. Cell division in both forms results in the production of two symmetrical cells. Both organisms are observed in fresh water and soil environments, particularly in eutrophic systems (Hirsch & Schlesner, 1981).

Angulomicrobium (Vasileva et al., 1979) is the generic name proposed for the triangular (Lafitskaya & Vasileva, 1976) and mushroom-shaped (Whittenbury & Nicoll, 1971) bacteria observed in fresh water and soil.

Metallogenium is an unusual bacterium that oxidises manganese (Zavarzin, 1981). It has been little studied and appears to have a complex life cycle. It produces coccoid cells and structures resembling prosthecae which lead to the development of rosettes and ultimately complex microcolonies.

1.4.7) The photosynthetic budding bacteria

These organisms are all purple non-sulphur bacteria and are classified with R. vannielii in the family Rhodospirillaceae, although evidence suggests (Imhoff et al., 1984) that this is not a phylogenetically natural grouping. Unlike Rhodomicrobium, many forms have been physiologically well studied and are capable of a variety of aerobic and anaerobic phototrophic and chemotrophic growth modes (Cox et al., 1983). Many of the Rhodospirillaceae reproduce by budding and are of particular interest because

they possess a cell cycle involving two cell types. The clearest example of this is in Rhodopseudomonas palustris (Whittenbury & McLee, 1967; Westmacott & Primose, 1976; Fig. 1.2) where a prostheca-like structure is produced, clear morphological differences exist between the mother and daughter cells and subsequent development of the two cells involves distinct mother and swarmer cell cycles. In Rhodobacter sphaeroides strain "Cordata", budding results in the production of cells of similar size and morphology but with the daughter cell possessing a prostheca and demonstrating a longer cell cycle than that of its mother (Gest et al., 1983). Rhodopseudomonas blastica (Eckersley & Dow, 1980) produces two morphologically identical cells at division which are physiologically distinct. The mother cell can initiate another round of reproduction immediately whereas the daughter cell must undergo a temporally variable period of environmentally controlled maturation before it can commence reproduction. Similar effects may occur in other members of this family whose growth cycles have yet to be characterised. We can see in this group of organisms a distinct gradient of morphological patterns (Fig. 1.7) all of which involve the production of swarmer-type cells which, as is the case in the prosthecate bacteria, only develop when environmental conditions are suitable.

1.4.8) The non-photosynthetic budding bacteria

The genus Pasteuria was proposed by Metchnikoff (1888; cited by Staley, 1981b) to incorporate a bacterium,

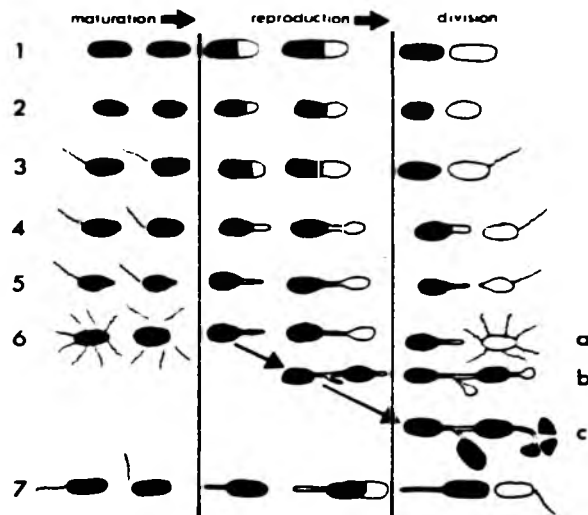


FIGURE 1.7 - Morphogenetic gradient of budding and prosthecate budding bacteria. The cell cycles are characterised as having three distinct stages: maturation, which may be temporally variable due to environmental factors, reproduction and cell division. The organisms illustrated are: (1) Escherichia coli growing with a doubling time of in excess of 60 minutes. (2) Rhodopseudomonas blastica. (3) Rhodopseudomonas acidophila. (4) Rhodopseudomonas palustris. (5) Hyphomicrobium. (6) Rhodomicrobium vannielii undergoing (a) the simplified cell cycle, (b) chain formation and (c) exospore production. (7) Caulobacter. Figure modified from Kelly & Dow (1984).

P. ramosa, which was observed to be parasitic on fresh water Daphnia and related crustaceans but was never isolated. Staley (1972) isolated two strains of budding bacteria from oligotrophic fresh water which were morphologically similar to those originally described by Metchnikoff and proposed that one of the isolates be designated a neotype strain. However, the original description of the genus made no mention of budding by the organism and the taxonomic position of Pasteuria ramosa sensu Staley remained enigmatic. In the intervening period Zavarzin (1961; cited by Hirsch, 1981a) had isolated organisms from oligotrophic fresh water morphologically very similar to those of Staley (1972) but had assigned them to a new genus Blastobacter since he was of the opinion that the absence of any description of budding by Metchnikoff (1888) disqualified his isolates from the genus Pasteuria. In the light of the recent isolation of a bacterial parasite of Daphnia identical to that described by Metchnikoff (Sayre et al., 1979), the generic name Pasteuria is not valid for budding bacteria of the type described in low nutrient environments by Staley (1972). The latter isolate has been reclassified in a new genus as Pirella staley (Schlesner & Hirsch, 1984) which is differentiated from Blastobacter (Sly, 1985) and a recent isolate of a budding bacterium from oligotrophic fresh water, Gemmata obscuriglobus (Franzmann & Skerman, 1984), on the basis of morphological, physiological and molecular criteria. All three genera are obligate budding bacteria which produce

holdfast material at the non-budding pole of the cell and to date have been found solely in low nutrient environments. In the light of the evidence that Planctomyces spp. do not synthesise stalks under "high" nutrient conditions (Schmidt & Starr, 1978), differentiation of Planctomyces from these budding genera on morphological grounds in natural systems is ill advised.

Members of the genus Seliberia are common in the low nutrient environment and are readily distinguished by virtue of their characteristic tight spiral morphology. Growth is by budding with the production of motile coccoid swarmer-type cells (Schmidt & Starr, 1984). The genus Nevskia consists of budding rods which produce an extracellular polysaccharide often in a form resembling a stalk (Hirsch, 1981b). The genera Caulococcus and Kusnezovia are budding cocci that have been observed in, but never isolated from, metal-rich environments (Schmidt & Zavarzin, 1981). Agromonas oligotrophica (Ohta & Hattori, 1983) is a budding rod-shaped nitrogen-fixing bacterium isolated from soil. Blastococcus aggregatus (Ahrens & Moll, 1970) is an unusual budding bacterium isolated from sea water. Cells divide to produce microcolonies and occasionally smaller flagellated vibrioid cells are produced which presumably function in dispersal.

1.4.9) How ubiquitous is unidirectional polar growth?

As discussed above budding growth is common in prosthecate, stalked and non-prosthecate bacteria isolated from low

nutrient environments and is often apparently associated with differential cell type expression. However, such a growth pattern is by no means limited to bacteria in such environments. A range of physiological types of bacteria exhibit unidirectional polar growth, for example Nitrobacter, Methylosinus, Siderococcus (Hirsch, 1974), the bacterial predator Ensifer (Casida, 1982) and the anaerobe Gemmiger formicilis isolated from faecal material (Gosling & Moore, 1975). Furthermore, as mentioned above, Escherichia coli cultivated at doubling times of in excess of 60 minutes appears to grow polarly (Donachie & Begg, 1970; Begg & Donachie, 1973; 1977), which is presumably a function of the inability of the organism to maintain more than one active growth point under limiting conditions (Kelly & Dow, 1984). Indeed, polar growth is an apparently ubiquitous phenomenon among bacteria and the prosthecae bacteria may merely represent extremes of expression (Fig. 1.7; Kelly & Dow, 1984).

Polarity in bacteria is by no means restricted to cell growth and the positioning of flagella and pili. The production of endospores in many Bacillus spp. can be considered as a modified asymmetric pattern of cell division, since it involves the asymmetric location of a septum and the subsequent production of the spore material excentrally (Fig. 1.8; Hitchins & Slepecky, 1969). Similarly, the asymmetric location of extracellular material, for example the trifoliin A-binding capsule of Rhizobium trifolii (Dazzo et al., 1982), is another clear example of cell

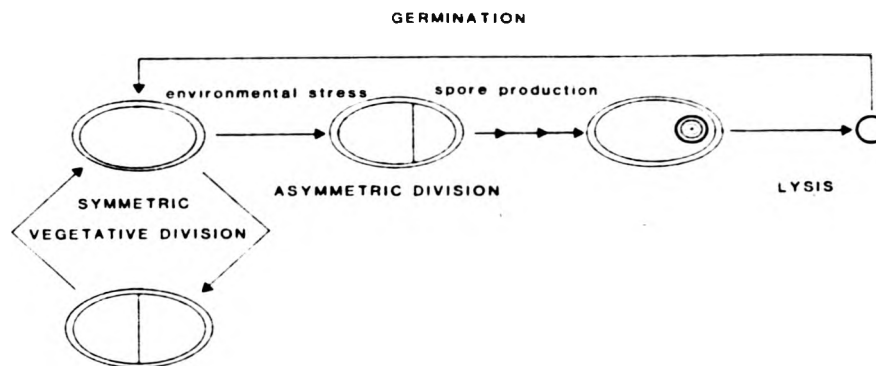


FIGURE 1.8 - Sporulation of *Bacillus subtilis* as a modified form of asymmetric cell division (concept of Hitchins & Slepecky, 1969). Under optimal conditions a standard vegetative cell cycle involving symmetric cell growth and division occurs. Under conditions of environmental stress an asymmetrically located septum is produced followed by the production of an endospore.

polarity. Evidence therefore suggests that expression of asymmetry in bacteria is by no means unusual nor is it restricted to "unusual" bacteria in "extreme" environments.

1.4.10) Consequences of the dimorphic cell cycle

The basic cell cycle of the prosthecate, stalked and many of the non-prosthecate budding bacteria can be summarised as illustrated in Figure 1.9. Stage A⁻ represents a metabolically restricted swarmer cell whose development is prevented by environmental conditions. Once conditions are suitable for reproduction to occur "inhibition" on the swarmer cell is removed (A) and an obligate period of maturation occurs resulting in the production of a reproductively competent cell (B) which can elongate by polar growth to produce a predivisional cell (C). Asymmetric division then normally occurs to yield an immature swarmer cell whose development may again be inhibited by environmental constraints (A⁻) and a reproductively competent mother cell (B). Not only, therefore, is there the possibility of aging in the mother cell (Whittenbury & Dow, 1977) but the swarmer cell, as a consequence of its ability to respond to the environment, can function as a survival and dispersal cell enabling escape from hostile environmental conditions (Dow *et al.*, 1983). As discussed above, the expression of polarity in bacterial cells potentially, and in many cases actually, results in differential cell type expression of this kind.

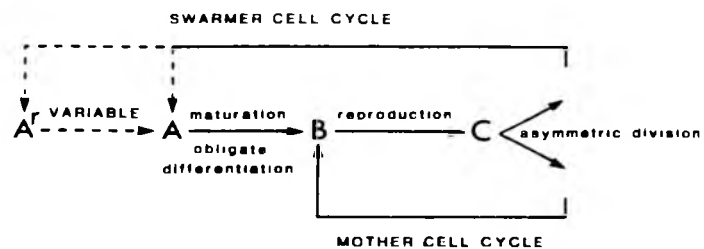


FIGURE 1.9 - Generalised cell cycle of prosthecate budding bacteria. For details see text. Figure from Morgan & Dow, 1985.

1.5) THE SWARMER CELL CONCEPT

1.5.1) The swarmer cell of prosthecate bacteria

As has been discussed for individual groups of prosthecate bacteria, the swarmer cell displays a limited metabolic activity, in most cases is actively motile and only initiates differentiation when environmental conditions are suitable for growth. In the light of these observations the concept has arisen that the swarmer cells are not only metabolically quiescent obligate stages in the cell cycles of these bacteria but serve a significant role in their ecology by functioning as survival and dispersal cells (Dow et al., 1983). The physiology of the swarmer cell and its ability to sense and respond to its environment are both important factors in this concept. Porter (1984) has shown that populations of swarmer cells of Rhodomicrobium vannielii maintained in darkness remain virtually 100% viable for 24 hours but there is a rapid decline in viability thereafter. Such a response is indicative of a short term survival role for the cell type, although in ecological terms it is necessary that only a small proportion of a swarmer cell population survives over a longer period of time for the propagation of the species. However, the swarmer cell sensu stricto as produced by the prosthecate bacteria is by no means unique in the bacterial world, although it can be considered as being unusual in its visibility. *I intend to discuss in the forthcoming pages the occurrence of motile dispersal stages and metabolically quiescent cell types in a range of "typical" and "atypical"*

bacteria and hope to demonstrate that the swarmer cell concept, as presently applied to the prosthecate bacteria, in fact holds true for many bacteria which respond to extreme nutrient limitation and other environmental stresses by the production of analogous quiescent cell types.

1.5.2) Other bacteria exhibiting differential cell type expression

One of the best documented cases of a bacterium producing a specialised survival and dispersal cell occurs in the bacterial parasite Bdellovibrio (Fig. 1.10; Thomashow & Rittenberg, 1979). The actively motile free-living vibrioid cell exists until a suitable host cell is encountered when the flagellum is shed and the host cell penetrated. There follows a period of cell growth and reproduction terminating in lysis of the host cell and release of a large number of vibrioid cells. In axenic laboratory culture differentiation of the vibrioid cell can be induced and in this case cell development does not occur until the flagellum is shed (Burnham et al., 1970), a situation analogous to that occurring in many prosthecate and budding bacteria.

The sheathed bacteria Sphaerotilus and Leptothrix (Fig. 1.11; Dow & Whittenbury, 1980; Mulder & Deinema, 1981) produce motile swarmer-type cells which attach to surfaces under suitable environmental conditions and clearly function as dispersal stages. Similar behaviour is observed in the filamentous gliding bacteria Leucothrix and Thiothrix (Fig. 1.12; Harold & Stanier, 1955; Brock, 1981; Larkin &

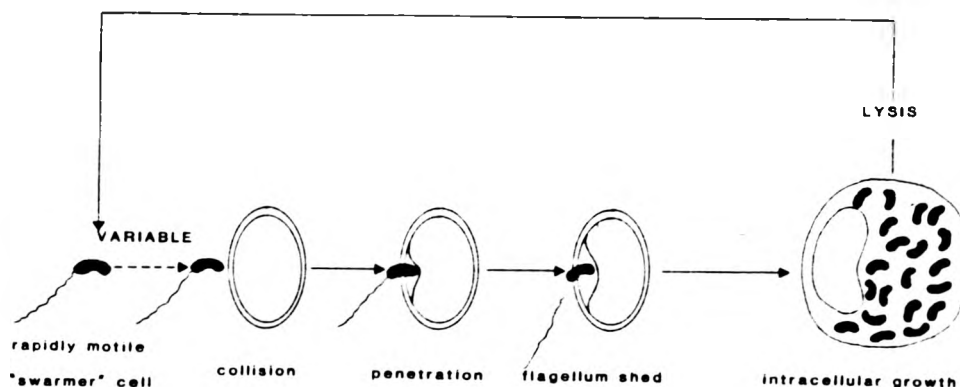


FIGURE 1.10 - Cell cycle of *Bdellovibrio*. The motile vibrioid cell encounters a host Gram-negative cell, penetrates the wall and loses its flagellum. Intracellular growth and division results in the production of large numbers of vibrioid cells which are released by lysis of the host cell and remain undifferentiated in the environment until they encounter a further host cell. After Thomashow & Rittenberg (1979).

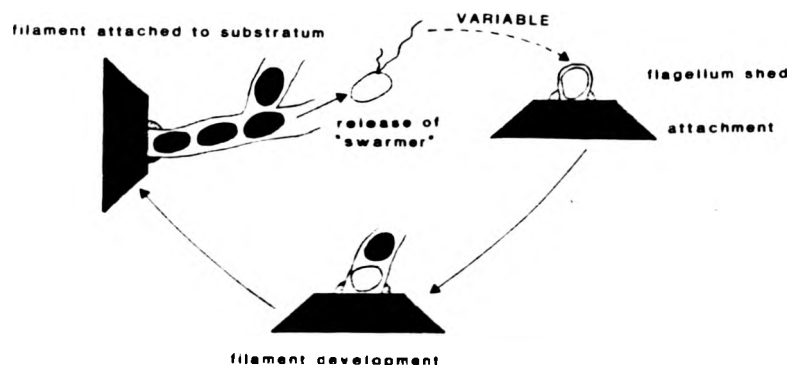


FIGURE 1.11 - Cell cycle of *Sphaerotilus*. The organism grows as filaments attached to surfaces and occasionally a flagellated cell is produced at the tip of the filament which is released. This cell remains free-living in the environment until a surface in a suitable environment is encountered. The flagellum is shed and holdfast synthesis results in attachment to the surface which is followed by cell growth, division and filament development. After Whittenbury & Dow (1980).

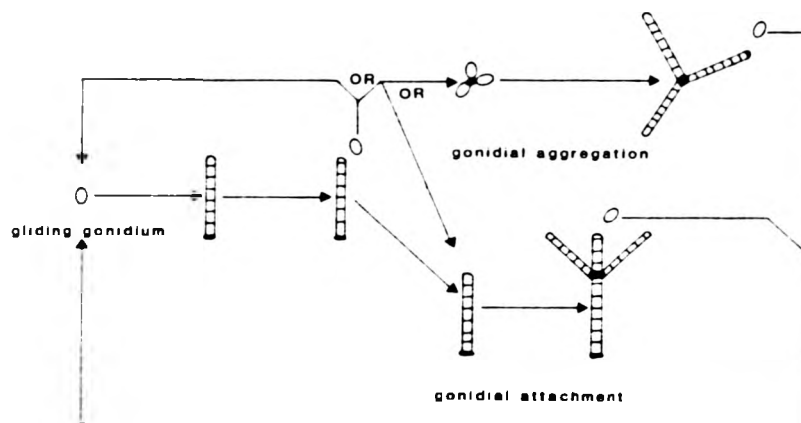


FIGURE 1.12 - Cell cycle of *Thiostrix nivea*. This sulphur-oxidising bacterium produces microcolonies with occasional production of gliding cells (gonidia) for dispersal which attach to surfaces prior to microcolony development (Larkin & Shinabarger, 1983). Complex developmental patterns can be observed depending on the pattern of gonidial development. A similar pathway occurs in the morphologically similar chemoheterotrophic bacterium *Leucothrix* (Brock, 1981).

Strohl, 1983; Larkin & Shinabarger, 1983) except that the motile cells (termed gonidia; Harold & Stanier, 1955) do not possess flagella and move by gliding. The coryneform bacterium Geodermatophilus exhibits a highly complex life cycle which is under environmental control (Fig. 1.13; Ishiguro & Wolfe, 1970; 1974). The motile R-form cells are maintained under nutrient limitation and may represent survival and dispersal stages.

In addition to the production of motile cells, some bacteria produce non-motile vegetative survival cells under adverse conditions. The trichomous bacterium Caryophanon (Trentini, 1978) produces thick walled cells termed sphaeroids which persist and remain viable under conditions which result in the death and lysis of the original trichome. The production of similar cell types in cyanobacteria (Dow et al., 1983) and myxobacteria (Dworkin, 1973) under certain conditions appears to be a similar response.

1.5.3) Bacterial dormancy

The concept of specialised survival cells is by no means new to microbial ecology nor does it always require the production of overtly distinct cell types. Stevenson (1978) has reviewed the concept of bacterial "exogenous" dormancy, "a condition in which development is delayed because of unfavourable chemical or physical conditions of the environment" (Sussman & Halvorson, 1966), and demonstrated that such an idea has long been proposed in the literature. The production of "dormant", metabolically quiescent cells by

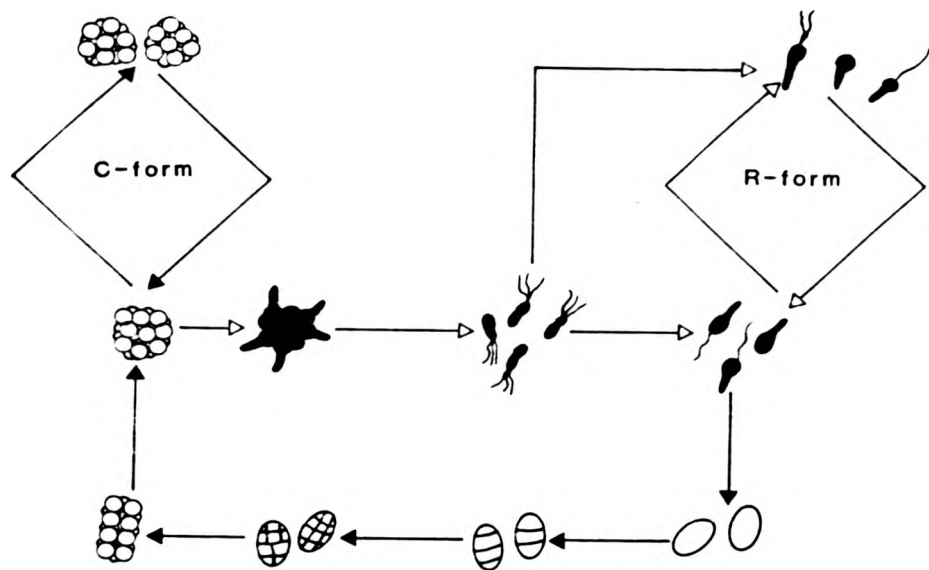


FIGURE 1.13 - Cell cycle of *Geodermatophilus*. Under comparatively nutrient-rich conditions (solid arrows) microcolony development occurs to produce the C-form of the organism which is maintained until nutrient stress (in particular, low ionic concentrations) ensues when the microcolony fragments (open arrows) to produce small actively motile cells (R-form). From Ishiguro & Wolfe, (1970; 1974).

bacteria under conditions of nutrient stress appears common. The best documented example is the decrease in cell size exhibited by many marine bacteria in response to starvation which results in the production of "ultramicrobacteria" (Torella & Morita, 1981; see Section 1.5.4). Apart from the size decrease no morphological changes occur in these organisms yet they are physiologically repressed and capable of long term survival. Similar responses have also been reported in a number of non-marine isolates (Morita, 1982; Stevenson, 1978).

The observation of very small bacterial cells in the natural environment does not, of course, prove their activity or viability in situ. Indirect evidence from laboratory studies (Morita, 1982) is merely circumstantial in such a respect and direct evidence is difficult to obtain. Using marine isolates in chemostat culture, Jannasch (1967) obtained evidence which suggested that a proportion of the population was viable yet dormant under conditions of nutrient stress. Vaccaro & Jannasch (1966) demonstrated that starved populations of marine bacteria responded too rapidly to the introduction of exogenous nutrients for this response to be due to de novo synthesis and this was probably due to the reactivation of dormant cells (Wright, 1973). Soil microbiologists have long proposed that the majority of bacteria in situ are dormant (see Gray & Williams, 1971; Stevenson, 1978) and the weight of evidence suggests that this is also the case in all oligotrophic environments.

1.5.4) The miniaturisation response of marine bacteria

The ubiquity of very small bacterial cells in the oceanic oligotrophic environment has been noted above (Section 1.3.2). The physiological nature of this type of cell and its production have been studied in the greatest detail in the Antarctic marine isolate Vibrio ANT-300. In laboratory culture under high nutrient conditions this organism grows normally producing morphologically typical vibrioid cells which, when harvested and resuspended in nutrient-free mineral salts solution, respond to starvation by fragmenting to produce large numbers of ultramicrocells (Novitsky & Morita, 1976). The initial effect of this process is a rapid increase in total viable count, often by ten orders of magnitude, but this is followed by a decrease in viable cells to a final level approximately equal to the original viability of the pre-starvation culture (Novitsky & Morita, 1977). The level of endogenous respiration in the viable cells is significantly curtailed (Novitsky & Morita, 1978). Initial breakdown of DNA, RNA and protein occurs during fragmentation but is followed by a slow but steady increase in the levels of these cellular components during the subsequent starvation period (Amy et al., 1983a). The precise identity of the energy sources for such anabolism are unknown but are believed to be lipid. In common with the swarmer cells of prosthecate budding bacteria ultramicrocells of ANT-300 possess a number of cell type-specific proteins of unknown function which are not found in the reproducing cell

(Amy & Morita, 1983a). Throughout starvation the ultramicrocell maintains an active high affinity arginine transport system (and presumably systems for other nutrients) which is dependent on proton motive force for its action (Geesey & Morita, 1979; Faquin & Oliver, 1984), whereas a low affinity system alone is produced by actively growing cells. So effective are the various adaptations to nutrient starvation in this organism that ultramicrocells of ANT-300 have been maintained in a viable condition in the laboratory for over 70 weeks (Novitsky & Morita, 1978). With relief from starvation comes cell growth and reproduction which occurs following a lag period whose length is proportional to the starvation period. Such a response has been monitored at the DNA, RNA and protein levels and is believed to represent recovery from a dormancy of increasing depth (Amy et al., 1983b).

A topic that has attracted particular interest in the light of evidence suggesting the importance of surface growth in situ is the effect of interfaces on the behaviour of starved marine bacteria. It has been shown that miniaturisation leads to an increased surface hydrophobicity of cells and a consequent increase in their affinity for surfaces (Kjelleberg & Hermansson, 1984). The importance of surfaces to starved marine bacteria clearly demonstrates how such a response is advantageous. Kjelleberg & Dahlback (1984) have shown that starved cells at interfaces have increased ATP levels in comparison to those in the bulk phase and Humphrey & Marshall (1984) have shown that surfaces stimulate

metabolic activity in ultramicrobacteria.

Behaviour analogous to that observed in ANT-300 and related strains occurs in a variety of native marine bacteria in laboratory culture (Torella & Morita, 1981; Jones & Rhodes-Roberts, 1981; Kurath & Morita, 1983; Amy & Morita, 1983b) and has also been observed in soil isolates (Nelson & Parkinson, 1978). In the latter context, the response of Arthrobacter to nutrient stress in continuous culture to produce miniaturised coccoid cells (Luscombe & Gray, 1974) may not be unrelated. Such evidence, in conjunction with the concepts of bacterial dormancy discussed above and the ubiquity of ultramicrobacteria in the natural environment (Fuhrman, 1981; Torella & Morita, 1981; Morita, 1982), clearly suggests that miniaturisation is highly important in the survival of bacteria in the oligotrophic natural environment, since it represents a "shut-down" survival and dispersal cell state. It is probable that the triggering levels of nutrients both into and out of the miniaturised state differs in populations in different trophic systems but, at present, evidence suggests that such quiescent cells play a major role in ensuring the survival and competitiveness of the native bacterial flora of at least the oceanic low nutrient environment.

It is hardly necessary to emphasise the functional and physiological similarities between ultramicrobacteria and the swarmer cells of prosthecate bacteria (Dow et al., 1983). Both appear to function, at least in part, as survival and dispersal cells and to have a similar physiological

nature. As a further, and possibly coincidental, demonstration of the resemblance between the two, it is interesting to note the behaviour of a marine vibrio strain at interfaces observed by Marshall (unpublished observations). On encountering a surface under low nutrient conditions the organism sheds its flagellum and attaches to the surface. If conditions are highly limiting the characteristic miniaturisation pattern is observed. However, if conditions permit, the bacterium divides to produce a daughter cell which is flagellated and therefore can function as a dispersal cell while the mother cell remains non-flagellated and attached to the surface.

1.5.5) How common are swarmer cells?

The evidence reviewed above strongly suggests that, at least in part, the swarmer cell concept as proposed by Dow et al. (1983) is not only valid for the prosthecate budding bacteria but that such cells occur in the majority of bacteria in the oligotrophic environment. However, Dow et al. (1983) also suggested that such a response might occur in those organisms familiar to most microbiologists as being characteristic of eutrophic environments and, in particular, in Escherichia coli, a model copiotroph (Hirsch, 1979), when growing under conditions more natural than those used for its cultivation in most laboratories. There is at least some physiological evidence for such a proposition, which not only goes some way to confirming this hypothesis but also demonstrates that responses to nutrient stress are not the

sole property of the "model" oligotroph but are, as I wish to emphasise, properties of nearly all microorganisms growing in the "most ubiquitous extreme environment" (Morgan & Dow, 1986), the low nutrient ecosystem.

If a morphologically typical organism were to produce a swarmer-type cell it would be expected that under conditions of nutrient stress there would be both a discontinuity in growth rate-dependent parameters below a critical growth rate and a response to nutrient pulsing too rapid to be accounted for by de novo synthesis (Morgan & Dow, 1985). The former has clearly been demonstrated in E. coli. It was proposed (Maałøe & Kjeldgaard, 1966) that the number of ribosomes in cells grown in continuous culture was directly proportional to the dilution rate, that is the cells contained only enough ribosomes for their needs at a given time. This was subsequently shown to be untrue below a critical dilution rate (Koch, 1971; Koch & Deppe, 1971) when E. coli contains an excess of ribosomes and utilises only a proportion of these at full efficiency and could therefore be considered as being prepared for an improvement in environmental conditions. More direct demonstrations of the potential existence of shut-down cells in E. coli are also available. Koch (1975) has shown that a culture grown to carbon exhaustion on very low concentrations of glucose is immediately viable on the addition of 0.02% glucose which is indicative of the ability of the organism to maintain itself in a viable quiescent state under starvation conditions. Furthermore, in a population growing at a low dilution rates

in continuous culture, only two thirds of cells were undergoing protein synthesis at any given time yet the whole culture was capable of de novo synthesis of β -galactosidase upon induction (Koch, 1979; Koch & Coffman, 1970).

There is therefore some basic physiological evidence for the existence of swarmer type cells in "ordinary" bacteria. It is therefore interesting to note evidence suggesting that there exist three growth rate-dependent growth modes in bacteria. These states have been detected in E. coli, Bacillus polymyxa and Paracoccus denitrificans (Table 1.3; Arbige & Chesbro, 1982; Chesbro et al., 1979; van Verseveld et al., 1984) and at doubling times of in excess of 50 hours it appears that a metabolically quiescent cell stage is formed. The importance of this response in bacteria in natural low nutrient environments and the recovery from this shut-down state have unfortunately not been investigated. However, all of these independent lines of evidence are highly suggestive that the swarmer cell concept (Dow et al., 1983) may hold true for a wide variety of bacteria.

1.6) PHYSIOLOGICAL ADAPTATIONS FOR GROWTH IN LOW NUTRIENT ENVIRONMENTS

To date I have considered the ecology of bacteria in the low nutrient natural environment and the potential importance of the swarmer cell state. In this section I intend to briefly

TABLE 1.3 - Characteristics of the three bacterial growth modes observed in continuous culture. After van Verseveld et al. (1984) where mode 1 was designated exploitation, mode 2 adaptation and mode 3 stringent control.

<u>Characteristic</u>	<u>1</u>	<u>2</u>	<u>3</u>
approx. t_d	min.-15h	33-50h	>100h
stringent response	basal	some?	full
storage compounds	accumulated	may use	used
cellular morphology	normal	may vary	may vary
cell size proportional to growth rate?	yes	need not be	need not be

review those physiological properties which, in addition to the potential production of metabolically quiescent swarmer-type cells, may enable bacteria to grow and compete successfully in the oligotrophic ecosystem.

1.6.1) Surface growth

The importance of interfaces in low nutrient environments was first clearly demonstrated by Zobell & Anderson (1936) who showed that the activity and numbers of bacteria in samples of sea water was proportional to the surface area available in the storage vessel. The physical concentration of ions and macromolecules at interfaces (Fletcher & Marshall,

1982; Wardell et al., 1983) accounts for the normal increase in bacterial biomass and activity observed at surfaces (reviewed by Fletcher & Marshall, 1982) although there is some evidence to show that this does not always occur (Bright & Fletcher, 1983). Certainly in the aquatic natural environments surface growth appears to be highly significant and has received a great deal of attention.

Initially the process of bacterial attachment to surfaces is a purely physical phenomenon (Wardell et al., 1983; Fletcher & Marshall, 1982). Subsequent successful attachment appears to depend on a variety of factors including the hydrophobicity of the cell envelope, the electrostatic properties of the cell and the production of extracellular adhesive polymers. Holdfast material is produced by a variety of bacteria (Fletcher & Marshall, 1982) and is found in many of the prosthecate bacteria commonly observed in oligotrophic environments attached to surfaces. The demonstration by Humphrey & Marshall (1984) that there is an increase in cell envelope hydrophobicity during miniaturisation of Vibrio DW1 also evidences the potential importance of surface attachment in oligotrophic systems. However, such information gives no information on the little studied topic of the subsequent development of communities at interfaces. These processes, which introduce the concept of a temporal parameter into microbial ecology, have aroused a good deal of interest (Fletcher & Marshall, 1982) and investigations should prove most interesting with respect to the competitiveness of organisms at interfaces in the low

nutrient environment.

1.6.2) Substrate-binding proteins

In Gram-negative bacteria there exist a variety of periplasmically-located proteins which possess no enzymological activity per se but bind to various substrates in the environment. Most investigations have involved E. coli, from which a variety of binding proteins have been isolated, including those for phosphate (Medveczky & Rosenberg, 1969), galactose (Anraku, 1967) and a variety of amino-acids (Rosen, 1971; Morita, 1982). These proteins are of considerable importance to bacteria in low nutrient ecosystems because of their threefold physiological roles in tactive responses, nutrient transport and environmental sensing (Morgan & Dow, 1986). Unfortunately no specific data are available on binding proteins in bacteria characteristic of oligotrophic environments.

1.6.3) Nutrient transport

One of the proposed characteristics of model oligotrophs (Hirsch, 1979) is that the majority of transport systems are constitutively synthesised and would therefore be immediately ready for use if a supply of nutrients suddenly became available. Such a response has been demonstrated in E. coli (Koch, 1979).

Bacteria growing under low nutrient conditions can effectively increase their nutrient uptake capacities by two means. The organism can increase the number of transport

sites in the membrane and consequently effectively increase the net maximum rate of transport. Alternatively, the organism can synthesise a transport system with a higher affinity for its substrate. The latter approach has been observed in a variety of bacteria for a variety of substrates, e.g. phosphate and certain amino-acids in E. coli (Rosenberg *et al.*, 1977; Rosen, 1971) and glucose in Pseudomonas aeruginosa (Harder & Dijkhuizen, 1983). Whilst there is little direct information available concerning the control of transport in bacteria characteristic of oligotrophic environments, there is a good deal of evidence showing that high efficiency of uptake is an important prerequisite for successful growth in the low nutrient environment (Matin & Veldkamp, 1978; Kuenen *et al.*, 1977; Akagi & Taga, 1980). In Vibrio ANT-300 high affinity uptake systems for amino-acids are only synthesised during miniaturisation (Geesey & Morita, 1979; Faquin & Oliver, 1984). Unfortunately nothing is known regarding the involvement of substrate-binding proteins in the transport processes in these organisms.

Similarly, little information is available concerning the transport systems of prosthecate bacteria. The existence of both high and low affinity glucose transport systems in prosthecae of Asticcacaulis biprosthecum has been demonstrated (Larson & Pate, 1976) and uptake of methylamine by Hyphomicrobium X is by means of an inducible system (Brooke & Attwood, 1984). It has long been proposed that the elongation of prosthecae with decreasing nutrient

concentrations represents a method for increasing the surface area to volume ratio of these organisms (Schmidt & Stanier, 1966) and such a response coupled with the production of high affinity nutrient transport proteins would significantly increase the uptake efficiency of these organisms.

1.6.4) Motility and taxes

The potential advantages of being chemotactic in the low nutrient environment are self evident and have been demonstrated in laboratory culture (Pilgram & Williams, 1976; Smith & Doetsch, 1969). It has been calculated that an actively respiring bacterium utilises approximately 0.1% of its available energy to operate one flagellum (Rowbury et al., 1983) and under conditions of nutrient stress the energy drain due to motility will be proportionally higher. If this is the case, it is not surprising that in the absence of nutrient gradients motility makes an organism comparatively uncompetitive with respect to non-motile cells (Pilgram & Williams, 1976; Rowbury et al., 1983). However, some workers are of the opinion that the operation of flagella make no energy demands of the cell other than that required to maintain the proton motive force (Harder et al., 1984).

In the majority of static low nutrient environments it is probable that gradients will be common and that the ability to move towards a local higher concentration of nutrients will be a great competitive advantage. Once again, it is unfortunate that oligotrophic bacteria have not been

investigated in detail for tactic responses. Of the prosthecate bacteria, only the swarmer cells of Caulobacter vibrioides have been investigated and have been shown to be chemotactic to a variety of amino-acids (Matveyeva & Gromov, 1983). The behaviour of ANT-300 is more interesting, for it is only after two to three days of starvation that chemotactic responses to a range of amino-acids, carbohydrates and other organic compounds can be detected (Geesey & Morita, 1979; Morita, 1982).

1.6.5) Intermediary metabolism

Studies on a variety of bacteria in continuous culture have yielded a large amount of information concerning the responses of many organisms to nutrient stress (for reviews see Dawes, 1976; Harder & Dijkhuizen, 1983; Tempest & Neijssel, 1978). Indeed, it is only necessary to consider the physiological properties of the aquatic Spirillum of Marin & Veldkamp (1978) that enabled it to outcompete a Pseudomonas in continuous culture to appreciate that fundamental physiological properties play an important role in ensuring an organisms ability to compete under conditions of nutrient stress. In this case a more efficient respiratory system, a lower minimum growth rate and a lower energy of maintenance all contributed to the organism's success. However, in the natural environment the situation is unlikely to be so simple, since it is likely that multi-factor limitation occurs. As an illustration of the complexities of investigating the physiology of cells under such

conditions it is merely necessary to consider the observations of Harder & Dijkhuizen (1983) who grew Pseudomonas oxalaticus in carbon-limited continuous culture on a mixture of oxalate and acetate. At dilution rates below 0.15h^{-1} the organism utilised both compounds equally but at dilution rates between 0.15 and 0.30h^{-1} acetate was utilised preferentially with excess oxalate remaining in the medium at a concentration proportional to the dilution rate. As an example of an organism that dramatically alters its intermediary metabolism in response to nutrient stress it is only necessary to consider the case in ANT-300 which displays a respiration rate of 0.0071% of its original level after seven days starvation.

A further complication to the picture of the physiology of a population under conditions of extreme nutrient limitation is the potential production of metabolically quiescent cells (Koch, 1979; van Verseveld et al., 1984) which will result in the formation of a heterogeneous population. Any investigation into the physiology of such a population or the physiology of a heterogeneous culture of prosthecae bacteria will in fact yield results that are the means of the two (or more) cell type populations present.

1.6.6) Nutrient storage

A wide variety of bacteria are known to accumulate excess nutrients in times of glut and store them as intracellular polysaccharides. Both phosphate (as PP_i) and carbon (as PHB,

lipids or carbohydrates) can be stored by organisms from low nutrient environments. It is important to note that the efficiency of such compounds in ensuring long-term survival under starvation conditions depends on their relatively slow utilisation (Dawes, 1976).

1.6.7) Others

A number of other metabolic factors may be involved in ensuring bacterial survival and growth in oligotrophic environments. I have deliberately chosen to ignore spore production in all its guises although its potential for survival is undeniable. More relevant to vegetative cells is the maintenance of the proton motive force across the cell membrane which is involved in nutrient uptake systems, cell motility and energy generation. It has been proposed that it is the inability of cells to maintain a proton motive force during nutrient stress that ultimately results in cell death (Harder *et al.*, 1984). In the light of the ability of certain bacteria to "block" their transport systems and hence prevent nutrient efflux when they are unable to prevent a significant decrease in the proton motive force (Konings & Veldkamp, 1983), such a topic is in need of detailed investigation.

As a final potential adaptation for growth in oligotrophic environments it is necessary to return to the topic of cell flotation. This may not only play a role in ensuring that the cell maintains itself in an aerobic environment but may serve to hold the cell in the

comparatively nutrient rich photic zone.

1.7) AIMS OF PROJECT

The overall aim of this project was to investigate selected prosthecate bacteria from a physiological point of view and attempt to relate these observations to their primarily oligotrophic mode of existence. Three lines of investigation were employed. Firstly, examination of samples from oligotrophic ecosystems was undertaken by direct observation and cultural means with the intention of ascertaining the nature of the bacterial flora therein and examining the importance of prosthecate bacteria. Secondly, investigations into the responses of prosthecate bacteria to nutrient limitation were performed in order to obtain some indication of the roles of prosthecae and swarmer cells in the natural environment. Thirdly, a number of enzymes of intermediary metabolism were examined in heterogeneous and swarmer cell populations of prosthecate bacteria with the aim of investigating the physiology of "model" oligotrophs and differential metabolic activity in prosthecate and swarmer cells.

PART 2 - MATERIALS AND METHODS

2.1) ORGANISMS AND BASIC CULTURE TECHNIQUES

2.1.1) Organisms

The bacteria used in this study were Caulobacter crescentus CB15, Rhodomicrobium vannielii Rm5 (Whittenbury & Dow, 1977), Hyphomicrobium X (Attwood & Harder, 1972), Rhodopseudomonas blastica (Eckersley & Dow, 1980), Rhodopseudomonas palustris, Escherichia coli C and Pseudomonas putida. Stock cultures in glycerol were obtained from the laboratory culture collection and stored at -80°C. Working cultures of R. vannielii and Rhodopseudomonas spp. were maintained in the appropriate liquid media at room temperature. Working cultures of all other bacteria were maintained on agar-solidified (Difco Bacto-agar; 15g l⁻¹) slopes of the appropriate media and were stored at 4°C. Culture purity was routinely checked by phase contrast microscopy.

2.1.2) Cultivation of Caulobacter

C. crescentus was cultivated aerobically at 30°C in Hutner's minimal medium (Poindexter, 1981b) containing 0.02% (w/v) glucose as the sole carbon source (HMG). The basal medium contained (per litre of distilled water):

Na ₂ HPO ₄ · 2H ₂ O	0.87g
KH ₂ PO ₄	0.53g
NH ₄ Cl	0.50g

The pH was adjusted to 7.0 and the medium sterilised by

autoclaving. Immediately prior to use the appropriate volume of sterile glucose solution and 10ml l⁻¹ of filter sterilised Hutner's concentrated base were aseptically added. Hutner's concentrated base contained (per litre of distilled water):

nitrilotriacetic acid	20.0g
KOH	14.6g
MgSO ₄ , 7H ₂ O	54.5g
CaCl ₂	6.7g
FeSO ₄ , 7H ₂ O	0.2g
(NH ₄) ₄ Mo ₇ O ₂₄ , 4H ₂ O	18.5mg
"metals 44" solution	100ml

Metals 44 solution contained (per litre of distilled water):

EDTA-Na ₂	2.50g
ZnSO ₄ , 7H ₂ O	10.95g
FeSO ₄ , 7H ₂ O	5.00g
MnSO ₄ , H ₂ O	1.54g
CuSO ₄ , 5H ₂ O	0.39g
Co(NO ₃) ₂ , 6H ₂ O	0.25g
Na ₂ B ₄ O ₇ , 10H ₂ O	0.18g

2.1.3) Cultivation of *Hyphomicrobium*

The Hyphomicrobium medium (HM) of Attwood & Harder (1972) containing 100mM methylamine as the sole carbon and energy source was routinely used. The organism was cultivated aerobically at 30°C. The basal salts solution of the medium contained (per litre of distilled water):

KNO ₃	5.00g
NaH ₂ PO ₄ , 2H ₂ O	1.38g
K ₂ HPO ₄	1.74g
(NH ₄) ₂ SO ₄	0.50g
MgSO ₄ , 7H ₂ O	0.20g

The pH was adjusted to 7.0 and the medium sterilised by autoclaving. Prior to inoculation the following were aseptically added from filter sterilised stock solutions: methylamine hydrochloride to a final concentration of 100mM; CaCl_2 to a final concentration of 0.025mg l^{-1} ; FeCl_2 to a final concentration of 3.5mg l^{-1} ; trace element solution, 0.5ml l^{-1} . Trace element solution contained (per litre of distilled water):

EDTA- Na_2	0.5g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.2g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	10mg
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	3mg
H_3BO_3	30mg
CaCl_2	20mg
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	1mg
$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	2mg
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	3mg

2.1.4) Cultivation of *Rhodospirillum rubrum* and

Rhodopseudomonas

R. vannielii was cultivated in 100ml, 5l, 10l and 20l batches in the pyruvate-malate medium (PM) of Whittenbury & Dow (1977). The basal medium contained (per litre of distilled water):

NH_4Cl	0.5g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.4g
NaCl	0.4g
sodium pyruvate	1.5g
sodium hydrogen malate	1.5g
CaCl_2	50mg

For the cultivation of *Rhodopseudomonas* spp. yeast extract

(Dxoid) was added at a concentration of 0.4g l^{-1} (PMYE). The pH was adjusted to 6.9, the medium dispensed into suitable vessels and sterilised by autoclaving. Prior to inoculation 50ml l^{-1} of sterile 100mM sodium phosphate buffer (pH 6.9) was aseptically added and the culture vessels stoppered with sterile rubber Suba-seals (W. Freeman & Co., Barnsley). The culture vessels were flushed for between 10 and 60 minutes (according to the vessel volume) with oxygen-free nitrogen (British Oxygen Co.) and then inoculated by injection through a Suba-seal. Cultures were incubated at 30°C with agitation on a rotary shaker (100ml volumes) or by means of a magnetic stirrer (larger volumes) with a light intensity of approximately 1000 lux.

Special photoheterotrophic cultures of R. vanniellii were grown anaerobically as described above in PM modified by the omission of pyruvate and malate which were replaced as carbon sources by 1.5g l^{-1} sodium acetate and a final concentration of 5mM NaHCO_3 . Microaerophilic cultures of R. vanniellii were grown in darkness at 30°C in 2l volumes of PM in cotton wool-plugged 2l conical flasks on a rotary shaker operating at 175 revolutions per minute.

2.1.5) Cultivation of *Escherichia coli* and *Pseudomonas putida*

These organisms were cultivated aerobically at 30°C in M9 mineral salts medium (Lark et al., 1963) containing 0.02% (w/v) glucose or 0.02% (w/v) sodium acetate as the sole carbon source. The medium contained (per litre of distilled

water):

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	7.0g
KH_2PO_4	3.0g
NaCl	0.5g
NH_4Cl	1.0g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2g
CaCl_2	20mg

The pH was adjusted to 7.0 and the medium sterilised by autoclaving. Carbon sources were added aseptically from sterile solution immediately prior to use.

2.2) GENERAL TECHNIQUES

2.2.1) Light microscopy

An Olympus PM-6 phase contrast microscope operating at a magnification of 1000 times was used. For photography an Olympus EMM-6 microscope camera system was attached and photographs taken on Kodak Panatomic-X film which was developed in Kodak D-19 developer and fixed in Kodak Kodafix fixer according to the manufacturers' instructions.

2.2.2) Electron microscopy

Formvar-coated copper grids (100 segment mesh; 3.05mm diameter; Agar Aida) were employed for specimen support. A drop of specimen suspension was placed on the grids and excess suspension removed by means of a strip of filter paper. The grid was allowed to dry and specimens either negatively stained or metal shadowed. Negative

staining was performed by placing a drop of 1% (w/v) phosphotungstic acid (pH 7.0) onto the grid and immediately removing all traces of the stain solution with a strip of filter paper. Gold-palladium metal shadowing was performed in an Edwards model E306A rotary shadower. Specimens were examined using a Jeol JEM-100S transmission electron microscope operating at an accelerating voltage of 80kV. Photographs were taken on Kodak 4489 Estar thick base electron microscope film which was developed in Kodak D-19 developer and fixed in Kodak Kodafix fixer according to the manufacturers' instructions.

2.2.3) Spectrophotometry

Routine determinations of culture optical density were performed at a wavelength of 600nm through a 1cm light path using a Pye Unicam SP500 series 2 spectrophotometer. More specialised data were obtained at the relevant wavelength using the above spectrophotometer or a Gilford model 250 linked to a Servoscribe chart recorder.

2.2.4) Electronic particle size analysis

A Coulter Counter model ZBI-C1000 Channelyzer system was employed for the determination of total cell counts and cell volume distribution analysis. The system was used at 1/amplification settings of 0.125, 0.25 or 0.5 selected as appropriate and the following standard settings: orifice diameter, 30µm; base channel threshold, 15; window width, 100; 1/current, 0.5. Cells for analysis were diluted in

Isoton (Coulter Electronics) to give a total cell count of between 20,000 and 80,000 and cell count data presented is the mean of a minimum of six counts. Detailed analyses of cell volume distribution data were performed by means of an Acorn BBC model B microcomputer running in-house software linked to the Channelyzer.

2.2.5) Synchronisation of *Rhodospirillum rubrum*

Late exponential phase cultures of *R. vannielii* (A₄₀₀ circa 1.0) were examined microscopically to ensure that they contained a suitable proportion of motile swarmer cells and were synchronised by passage through glass wool columns as described by Whittenbury & Dow (1977; Fig. 2.1). The degree of synchronisation was checked by direct microscopic examination and by analysis using the Coulter Counter. Swarmer cells were maintained in darkness at room temperature for the minimum possible period before being utilised.

2.2.6) Continuous culture

New Brunswick BioFlo C30 chemostats operating at 30°C with a working volume of approximately 320ml were employed for all studies. Aeration was provided by the inbuilt air pumps operating at maximum rate and agitation was provided by means of the inbuilt magnetic stirrer operating at 500 revolutions per minute. Oxygen tension and pH were monitored by means of model 40 or model 400 control units and the pH adjusted if necessary by the automated addition of 0.1M NaOH. Medium was added by means of the inbuilt pump or a Uniscil MP-3

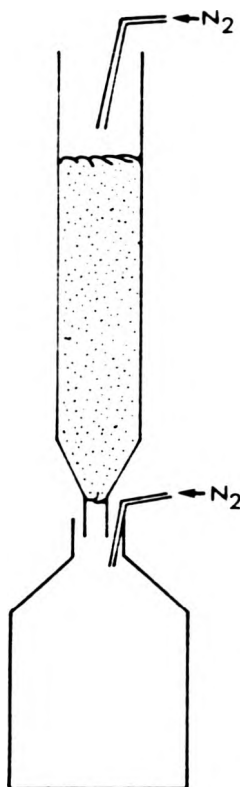


FIGURE 2.1 - Apparatus for the synchronisation of *Rhodospirillum rubrum* (method of Whittenbury & Dow, 1977). Glass columns of suitable size were loosely packed with glass wool and sterilised by autoclaving. The column was covered in aluminium foil to maintain the interior in darkness, rinsed through with sterile distilled water to remove debris and a sterile, foil-covered collecting vessel placed underneath. Both the column and the collecting vessel were flushed with oxygen-free nitrogen for 30 minutes before the culture was poured slowly into the top of the column. Effluent from the bottom of the column contained a synchronised population of swarmer cells and the degree of synchrony was monitored by microscopic examination and Coulter Counter analysis.

peristaltic pump.

Following sterilisation the system was equilibrated with respect to the controlled parameters and inoculated with approximately 5ml of a late exponential phase batch culture. Incubation was continued until exponential growth ensued and medium flow at the desired rate begun. The bacterial population was monitored for contamination by direct microscopic examination and by streaking samples onto plates containing an agar-solidified version of the medium in use. Cell density was monitored by measuring A_{400} and by total cell counts obtained using the Coulter Counter. The culture was deemed to be in steady state when these parameters remained unchanged over a period corresponding to in excess of three volume changes.

Maximum growth rate (μ_{max}) was determined in duplicate in each medium by means of the washout rate method described by Jannasch (1969). The culture was grown to steady state at a convenient high dilution rate (D) and the medium flow rate increased to give a dilution rate just below that expected to lead to wash out of the culture. The total cell count in the culture declines due to the increase in flow rate and this was measured by means of the Coulter Counter over a 24 hour period. Since

$$\ln(x_1) - \ln(x_2) = (D - \mu_{max})(t_2 - t_1)$$

(where x_1 and x_2 are the cell counts at times t_1 and t_2 respectively), a plot of $\ln(x)$ against time has a slope of $(D - \mu_{max})$, therefore enabling the value of μ_{max} to be determined (Tempest, 1970).

E. coli C was cultivated in carbon-limited continuous culture in M9 media containing 0.02% and 0.002% (w/v) glucose as the sole carbon and energy source. Hyphomicrobium X was cultivated in carbon-limited culture in HM prepared as described above and in phosphate-limited culture in a modified HM which was prepared as described above except that the addition of K_2HPO_4 was reduced to $87mg\ l^{-1}$ and that of $NaH_2PO_4 \cdot 2H_2O$ to $31mg\ l^{-1}$ (total inorganic phosphate concentration of 1mM). In all cases the identity of the limiting nutrient was determined by increasing the levels of the phosphate or carbon sources in the reservoir medium by an appropriate factor and observing a consequent increase in cell numbers in the culture vessel.

2.3) ECOLOGICAL TECHNIQUES

2.3.1) Water samples

Two distinct sources of oligotrophic water were sampled. Esthwaite Water, Cumbria is a stratified lake (hypolimnion at approximately 7.5m) from which samples were collected during September 1984. Four samples were collected: surface water, ambient temperature 22°C; 6m depth, ambient temperature 20°C; 10m depth, ambient temperature 13°C; circa 16m depth, ambient temperature 11°C. The sample from 16m depth was close to the lake bottom and contained a significant amount of sediment. The second source of water was a number of distilled and double distilled water storage vessels in the laboratory. These were 20l glass aspirator bottles,

stoppered with foam rubber and in which there were lengths of silicone tubing. The ambient temperature of these vessels ranged from approximately 18 to 26°C. Samples were taken of bulk water and of bacterial growth on surfaces, the latter by means of sterile cotton wool swabs.

2.3.2) Batch enrichment culture

Water samples were placed in sterile conical flasks and incubated in darkness with minimal disturbance at 15, 20 or 25°C, as appropriate to the sample source. To some samples a sterile stock solution of Difco Bacto-peptone was added to give a final concentration of 0.01% or 0.001% (w/v).

Enrichment cultures were sampled at intervals with the minimum disturbance. Samples of bulk water were removed with a sterile pipette, growth on the walls of the flask by means of a sterile cotton wool swab and surface pellicle by means of a sterile inoculation wire.

2.3.3) Continuous flow enrichment culture

The apparatus employed is illustrated in Figure 2.2. A New Brunswick BioFlo C30 chemostat system culture vessel was modified by the removal of the magnetic stirring vane and the shortening of the air inlet tube. To the vessel was added 75g of glass beads (2.5-3.5mm diameter; BDH) and the entire assembly sterilised by autoclaving. The culture vessel was mounted on the chemostat console and a medium reservoir and waste collection vessel aseptically attached. The vessel was filled with medium and equilibrated with respect to

temperature (25°C; this necessitated the use of both a heating element and a "cold-finger") and oxygen content (maximal air flow). Between 10 and 20ml of sample water from a double distilled water storage vessel was aseptically added and incubated for 48 hours. After this time medium was pumped through the system by means of a Uniscil MP-3 peristaltic pump at a flow rate of between 0.1 and 1.2ml min⁻¹. After four weeks incubation, samples of medium, glass beads and general bacterial growth on the wall of the vessel were aseptically taken and the vessel cleaned, sterilised and reinoculated from the same source.

Two media were employed: Difco Bacto-peptone (0.01% (w/v)) and the defined medium of Staley (1981a) containing 0.002% (w/v) glucose. The basal medium contained (per litre of distilled water):

(NH ₄) ₂ SO ₄	0.25g
Na ₂ HPO ₄ ·2H ₂ O	74mg

Following sterilisation by autoclaving, the following were aseptically added from sterile stock solutions: glucose to 0.002% (w/v); Hutner's concentrated base, 20ml l⁻¹; vitamin solution, 10ml l⁻¹. The vitamin solution (Staley, 1981a) contained (per litre of distilled water):

vitamin B ₁₂	0.1mg
biotin	2.0mg
calcium pantothenate	5.0mg
folic acid	2.0mg
nicotinamide	5.0mg
pyridoxine hydrochloride	10.0mg
riboflavin	5.0mg
thiamine hydrochloride	5.0mg

2.3.4) Media and techniques for bacterial isolation

Bulk phase samples from the natural environment and enrichment cultures were streaked either directly or following dilution by up to 1000 times in sterile double distilled water onto agar-solidified versions of the following media: 0.01% and 0.001% (w/v) Bacto-peptone, the defined medium of Staley (1981a) containing 0.002% (w/v) glucose, HM (all solidified with Difco Bacto-agar) and double distilled water solidified with Difco Noble agar (high purity, low in organic compounds). Samples on swabs were streaked directly onto such media whereas glass beads from continuous flow enrichment culture were briefly agitated in a small volume of sterile double distilled water using a vortex-mixer and the resulting cell suspension streaked onto the media. Plates were incubated for between 4 and 12 weeks at an appropriate temperature wrapped in perforated plastic cling-film to prevent them drying out during the incubation period. Following incubation, individual colonies were picked from plates by means of a sterile inoculation wire, streaked onto the appropriate medium and incubated as described above. Pure isolates were maintained at 4°C on slopes of the medium on which they had been isolated.

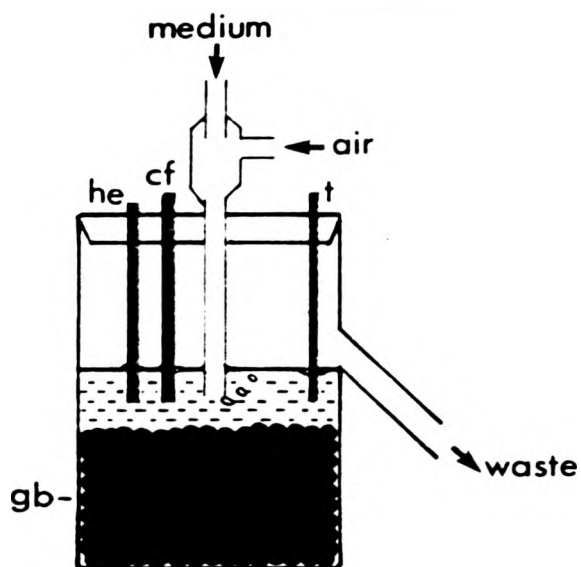


FIGURE 2.2 - Continuous flow culture system for the analysis of the bacterial flora of aquatic environments. For details see text. he, heating element; cf, cold finger; t, thermocouple; gb, glass beads. The volume of medium in the culture vessel was approximately 300 ml.

2.4) EXAMINATION OF NUTRIENT EFFECTS ON CELL MORPHOLOGY AND CELL TYPE EXPRESSION

2.4.1) Cultivation of organisms

The effects of variations in calcium, phosphate and organic carbon concentrations on Hyphomicrobium X, C. crescentus and R. vannielii were examined in modified versions of HM, HMG and PM, respectively. Media were prepared as described above except that phosphate, calcium and/or organic carbon components were omitted as appropriate. Prior to inoculation the omitted components were aseptically added from sterile stock solutions as desired. If the concentration of phosphate was to be decreased below the level normally present, the medium was supplemented with MOPS (3-[N-morpholino]propanesulphonic acid)-NaOH buffer (pH 6.9) to a final concentration of 10mM. Preliminary experiments demonstrated that MOPS had no inhibitory or stimulatory effect on any organism tested and could maintain the pH value of the medium at the desired value.

The effects of variation in the concentration of organic carbon on natural isolates, C. crescentus and E. coli were determined using a defined medium modified from that of Staley (1981a). To the sterile medium base the following were added immediately prior to inoculation: MOPS buffer (pH 6.9) to 10mM; Hutner's concentrated base, 1ml l⁻¹; vitamin solution (Staley, 1981a), 1ml l⁻¹; carbon source, as appropriate. The following compounds were used as carbon

sources: Bacto-peptone, glucose, sodium succinate, sodium citrate, galactose.

All cultures were incubated under the appropriate conditions until the stationary phase of growth had been reached as determined by measurements of A_{400} and total cell counts obtained using the Coulter Counter. Chromic acid-washed glassware was utilised at all times.

2.4.2) Examination of cellular morphology

General cell volume distribution data were obtained using the Coulter Counter ZBI-C1000 Channelyzer system as described above. For prosthecate bacteria these data were supplemented with measurements of prostheca length obtained from electron microscope preparations. The lengths of in excess of 100 prosthecae were measured either directly or on photographic prints and actual size determined by comparison to standard diameter latex spheres (Agar Aids).

2.4.3) Examination of cell type expression

Cell type expression of prosthecate bacteria was determined by direct cell type counts of in excess of 300 cells in electron microscope preparations. Cells were graded into three groups: swarmer (non-prosthecate) cells, reproducing cells and prosthecate non-reproducing cells. Results are expressed in terms of the ratios of swarmer cells to non-reproducing prosthecate cells and reproducing to non-reproducing prosthecate cells.

2.5) ANALYSIS OF RADIOLABELLING PATTERNS

2.5.1) Incorporation of radiolabel

Cultures (100ml) of R. vannielii, Rhodopseudomonas blastica and Rhodopseudomonas palustris were grown as described above in the presence of one of the following radiolabelled sodium salts: [U- ^{14}C]-acetate, $0.5\mu\text{Ci ml}^{-1}$; [1- ^{14}C]-acetate, $0.5\mu\text{Ci ml}^{-1}$; [2- ^{14}C]-acetate, $0.5\mu\text{Ci ml}^{-1}$; [U- ^{14}C]-malate, $0.1\mu\text{Ci ml}^{-1}$. Incorporation of radiolabel was monitored by the removal of 1ml samples from the cultures. These were filtered through $0.22\mu\text{m}$ pore size Millipore membrane filters, washed twice with 1ml portions of sterile medium, the filters placed in vials containing Beckman EP scintillation fluid and radioactivity determined using a LKB Minibeta 1212 liquid scintillation counter.

2.5.2) Purification and hydrolysis of total cellular protein

Portions of late exponential phase ($A_{400} = 1.0$) radiolabelled cultures were added to equal volumes of 10% (w/v) trichloroacetic acid and incubated on ice for 30 minutes. Insoluble material was pelleted by centrifugation at $18,000 \times g$ and 4°C for 15 minutes, resuspended in 10ml 70% (v/v) ethanol and incubated at 45°C for 45 minutes. The insoluble material was pelleted as described above, resuspended in 10ml diethyl ether-ethanol (1:1) and incubated at 30°C for 45 minutes. Insoluble material was pelleted as described above, resuspended in 10ml 5% (w/v) trichloroacetic acid and incubated at 95°C for 30 minutes. Insoluble material was

pelleted as described above, washed thrice in ethanol and resuspended in approximately 2ml 6M HCl. The suspension was sealed into glass vials and protein hydrolysed by incubation at 105°C for 18 hours. Hydrolysates were stored at -20°C until required.

2.5.3) Two-dimensional chromatography

Volumes (20 - 150µl) of radiolabelled protein hydrolysate samples were loaded onto Whatman No. 1 chromatography paper (20 x 20cm). Chromatograms were run in the first dimension for 16 hours in a solvent consisting of butanol-acetic acid-water (180:45:75) and in the second dimension for 15 hours in a solvent consisting of 80% (w/v) aqueous phenol-saturated ammonia solution (300:1.5). Chromatograms were air-dried and amino-acid spots visualised by spraying the chromatogram with 0.25% (w/v) ninhydrin dissolved in acetone and developing at 100°C for 2-3 minutes. The identity of individual amino-acid spots was ascertained by comparison with chromatograms of defined amino-acid mixtures (chromatographically pure amino-acids; BDH) run concurrently.

The levels of radioactivity in individual amino-acid spots were determined in two ways. Basic qualitative information was obtained by autoradiography. Fuji X-ray film was exposed to chromatograms for between two and four weeks, developed in Kodak LX-40 developer and fixed in Kodak FX-40 according to the manufacturers' instructions. Quantitative data were obtained by carefully cutting stained spots from individual chromatograms, placing them in scintillation vials

containing Beckman EP scintillation fluid and counting in an LKB Minibeta 1212 liquid scintillation counter.

2.6) ANALYTICAL TECHNIQUES

2.6.1) Analysis of intracellular polyphosphate

Portions (20ml) of early stationary phase cultures were harvested by centrifugation at $14,000 \times g$ and 4°C for 10 minutes and cells washed twice with sterile phosphate-free medium. Polyphosphate was purified using a method modified from that of Poindexter & Eley (1983). The washed cell pellet was resuspended in 1ml of alkaline hypochlorite reagent (10% (v/v) BDH "Spectrosol" sodium hypochlorite; pH 9.8; Williamson & Wilkinson, 1958) and incubated at 30°C for one hour. Insoluble material was pelleted by centrifugation in an Eppendorf Microfuge at 4°C for 10 minutes, resuspended in 1ml 1.5M NaCl solution containing 1.5mM EDTA- Na_2 and incubated at 20°C for 5 minutes. Insoluble material was pelleted as described above and resuspended in 800 μl of sterile distilled water. Concentrated HCl (200 μl) was added and polyphosphate hydrolysed at 100°C for 30 minutes. Samples were stored at -20°C until required.

The concentration of inorganic phosphate was determined by means of a Sigma inorganic phosphate assay kit (procedure 360-UV). This procedure involves measuring the absorbance at 340nm due to the production of a reduced phosphomolybdate complex in the following reactions:

$P_i + H_2SO_4 + \text{ammonium molybdate} \rightarrow \text{phosphomolybdate complex}$

Sigma reagent (1ml) was pipetted into 1ml quartz cuvettes and 10 μ l of sample added. The reaction was allowed to proceed for five minutes and the absorbance measured against a blank cuvette containing 1ml reagent plus 10 μ l sterile double distilled water. Actual phosphate concentration was determined from a standard calibration curve prepared for each experiment using standard phosphate solutions and expressed as a function of cellular protein. Chromic acid-washed glassware was employed at all times.

2.6.2) Preparation of cell-free extracts for enzyme assays

Cells were harvested from 5l (R. vannielii and Rhodopseudomonas palustris) or 1l (E. coli, P. putida and C. crescentus) late exponential phase cultures by centrifugation at 10,000 x g and 4°C for 20 minutes. Cells were washed twice in sterile medium and resuspended in approximately 20ml of sterile 10mM tris-HCl (pH 7.0) to produce a thick cell paste. Synchronous populations of R. vannielii swarmer cells were prepared from 10 or 20l cultures as described above and concentrated to a minimal volume by means of a Millipore Pellicon ultrafiltration system prior to centrifugation. All enzyme assays were performed within five hours of the preparation of the lysate.

Two methods of cell disruption were employed. Ultrasonication was performed using an MSE 12/76 mark 2 ultrasonicator operating at a peak to peak wavelength of

between 10 and 12 μ m. Cells were sonicated for a cumulative period of two minutes in 15 second pulses at one minute intervals cooled in a bath of methanol-ice. Passage through a French pressure cell was less commonly employed. Cell paste was passed through the cell twice at 20,000 pounds per square inch and 4°C. In both cases unbroken cells and cell debris were removed by centrifugation at 18,000 \times g and 4°C for 45 minutes and the lysates maintained on ice.

2.6.3) Assay of enzymes of the tricarboxylic acid cycle

TCA cycle enzymes were all assayed spectrophotometrically using variable concentrations of cell-free extract as appropriate to the individual assay. Quartz cuvettes of 3ml volume and 1cm light path were employed and all assays were performed in a total reaction volume of 3ml at room temperature. The enzymatic nature of the reactions was confirmed by means of suitable component-omission assays and protein concentration-dependent reaction rates demonstrated. Activity was calculated using the slopes of the initial linear portion of plots of absorbance against time.

Citrate synthase (citrate:oxaloacetate lyase (CoA acetylating); EC 4.1.3.7) was assayed according to the method of Reeves et al. (1971). The procedure involves measuring the increase in A_{412} as a consequence of the reaction of 5,5'-bisthiol(nitrobenzoic acid) (DTNB) with sulphhydryl groups on CoA-SH produced from acetyl-CoA in the conversion of oxaloacetate to citrate. The rate of reaction due to citrate synthase is determined from the slope of the

absorbance change in the presence of oxaloacetate minus that due to deacylase reactions in the absence of oxaloacetate.

The reaction mixture contained (μ moles in 3ml):

tris-HCl (pH 8.1)	600.0
potassium oxaloacetate	1.5
acetyl-CoA	0.3
DTNB	0.6

The reaction was started by the addition of oxaloacetate.

Aconitase (citrate (isocitrate) hydro-lyase; EC 4.2.1.3) was assayed in a coupled system by converting the isocitrate produced by the action of the enzyme on cis-aconitate or citrate to 2-oxoglutarate by means of exogenously supplied isocitrate dehydrogenase and measuring the change in absorbance at 340nm due to the production of NADPH by the latter enzyme. The reaction mixture contained (μ moles in 3ml):

tris-HCl (pH 7.5)	300.0
MnCl ₂	6.0
NADP	1.5
sodium cis-aconitate	15.0

plus 1 unit of isocitrate dehydrogenase

Sodium citrate was used in place of the cis-aconitate in certain experiments. The reaction was started by the addition of the citrate or cis-aconitate.

Isocitrate dehydrogenase (L₂-isocitrate:NAD oxidoreductase (decarboxylating); EC 1.1.1.41 and L₂-isocitrate:NADP oxidoreductase (decarboxylating); EC 1.1.1.42) was assayed according to the method of Reeves

et al. (1971) by measuring at 340nm the isocitrate-dependent reduction of NAD or NADP. The reaction mixture contained (μmoles in 3ml):

tris-HCl (pH 7.4)	60.0
MnCl ₂	2.0
NAD or NADP	0.5
sodium isocitrate	0.5

The reaction was started by the addition of isocitrate.

The 2-oxoglutarate dehydrogenase enzyme complex was assayed using a method modified from that of Reeves et al. (1971) involving measurement at 340nm of the 2-oxoglutarate-dependent reduction of NAD to NADH. The reaction mixture contained (μmoles in 3ml):

tris-HCl (pH 8.5)	500.0
potassium 2-oxoglutarate	25.0
NAD	6.0
L-cysteine	7.8
KCN	2.0
CoA	0.3

The reaction was started by the addition of 2-oxoglutarate.

Succinate thiokinase (succinate:CoA ligase (ADP); EC 6.2.1.5) was assayed according to the method of Cha (1969). This involved the coupling of the reverse reaction of the enzyme through pyruvate kinase (ATP:pyruvate phosphotransferase; EC 2.7.1.40) and lactate dehydrogenase (L-lactate:NAD oxidoreductase; EC 1.1.1.27) and measuring the production of NAD from NADH at 340nm due to the following sequence of reactions:

succinate thiokinase:

$\text{ATP} + \text{CoA} + \text{succinate} \rightleftharpoons \text{ADP} + \text{succinyl-CoA} + \text{P}_i$

pyruvate kinase:

$\text{ADP} + \text{phosphoenolpyruvate} \rightleftharpoons \text{pyruvate} + \text{ATP}$

lactate dehydrogenase:

$\text{pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{NAD}$

The rate of production of NAD from NADH is a sole function of succinate thiokinase activity once the background activities due to NADH and ATP-utilising reactions are subtracted. The reaction mixture contained ($\mu\text{moles in } 3\text{ml}$):

tris-succinate (pH 7.4)	150.0
ATP	0.7
CoA	0.3
potassium phosphoenolpyruvate	4.7
NADH	0.6
KCl	300.0
MgCl ₂	30.0

plus 50 μl of a solution of 2 mg ml⁻¹ each of lactate dehydrogenase and pyruvate kinase dissolved in 100mM tris-HCl buffer (pH 7.4)

The reaction was started by the addition of cell lysate.

Succinate dehydrogenase (succinate: (acceptor) oxidoreductase; EC 1.3.99.1) was assayed by measuring at 600nm the succinate-dependent reduction of 2,6-dichlorophenolindophenol (DCPIP) mediated by phenazine methosulphate (PMS). The assay system used was modified from that of King (1963) and contained ($\mu\text{moles in } 3\text{ml}$):

potassium phosphate (pH 7.6)	150.0
DCPIP	0.2
PMS	1.2
sodium succinate	20.0
KCN	7.0

The reaction was started by the addition of succinate.

Fumarase (L-malate hydro-lyase; EC 4.2.1.2) was assayed by the method of Reeves et al. (1971) involving the direct measurement at 300nm of fumarate utilisation. The reaction mixture contained (μ moles in 3ml):

potassium phosphate (pH 7.4)	100.0
potassium fumarate	25.0

The reaction was started by the addition of fumarate.

Malate dehydrogenase (L-malate:NAD oxidoreductase; EC 1.1.1.37) was assayed by measuring the malate-dependent oxidation of NAD at 340nm according to the method of Reeves et al. (1971). The reaction mixture contained (μ moles in 3ml):

potassium phosphate (pH 7.4)	85.0
potassium oxaloacetate	1.3
NADH	0.4

The reaction was started by the addition of oxaloacetate.

2.6.4) Assay of 2-oxoglutarate utilisation

The assay mixture contained (in 1ml):

tris-HCl (pH 8.5)	150.0μmol
L-cysteine	2.0μmol
CoA	0.1μmol
NAD	2.0μmol
[5- ¹⁴ C]-2-oxoglutarate	0.1μCi

The mixture was pre-incubated at 30°C for 10 minutes and a volume of R. vannielii or E. coli lysate added to give a protein concentration of approximately 7mg ml⁻¹. The reaction mixture was sampled at regular intervals and the reaction stopped by freezing 1ml portions of reaction mixture in liquid nitrogen. Samples were stored at -20°C until required and thawed on ice.

Separation of organic acids was by means of ascending paper chromatography on Whatman No.1 chromatography paper of 50μl assay samples in a solvent of butanol-acetic acid-water (60:15:25) with a run-time of 5 hours. The distribution of radioactivity along the chromatograms was determined by cutting them into 1cm strips which were counted by liquid scintillation means as described above. Positions of standard organic acids were determined on standard chromatograms run concurrently with the assay samples. Chromatograms were developed by immersion in a solution of bromophenol blue and methyl red (both at 1% (w/v)) in 95% (v/v) ethanol and organic acids appeared as yellow spots on a blue background.

2.6.5) Assay of enzymes of carbon dioxide fixation

Assay of CO₂ fixation in whole cells of R. vannielii was performed using late exponential phase cells resuspended in PM medium and incubated in the presence of radiolabelled CO₂

under the conditions used for cultivation. Cells (culture $A_{400} = 1.0$) were harvested by centrifugation at $14,000 \times g$ and 4°C for 15 minutes, washed twice in sterile medium and resuspended in sterile PM to give an A_{400} of approximately 0.3. Portions (10ml) of cell suspension were placed in 25ml conical flasks, the flasks stoppered with Suba-seals and flushed for 10 minutes with oxygen-free nitrogen. Cultures were equilibrated in a shaking water bath at 30°C with a light intensity of approximately 1000lux for 10 minutes and NaHCO_3 solution added to give a final concentration of 2.5mM. Incubation was continued for a further 10 minutes and the assay started by the addition of $10\mu\text{Ci NaH}^{14}\text{CO}_3$. Samples (1ml) were removed at intervals, added to 2ml of ethanol-acetic acid (95:5) and evaporated to dryness at 105°C . The residue was resuspended in approximately 200 μl of sterile double distilled water and 3ml of Beckman EP scintillation fluid added. Residual radioactivity was determined using a LKB Minibeta 1212 liquid scintillation counter (efficiency of counting = 90%).

D-ribulose-1,5-bisphosphate carboxylase-oxygenase (RUBISCO; 3-phospho-D-glycerate carboxy-lyase (dimerising); EC 4.1.1.39) was assayed using a method modified from that of Lorimer *et al.* (1977). Late exponential phase cells ($A_{400} = 1.0$) of *R. vannielii* were harvested and washed as described above, resuspended in a minimal volume of TEMMB buffer and cell-free extract prepared as described in Section 2.6.2. TEMMB buffer contained:

tris-HCl (pH 8.0)	20mM
MgCl ₂ ·6H ₂ O	10mM
NaHCO ₃	50mM
EDTA-Na ₂	1mM
2-mercaptoethanol	5mM

To 100µl of cell-free lysate was added 150µl 100mM tris-HCl (pH 8.2) containing 5µCi NaH¹⁴CO₃ and the assay mixture equilibrated at 30°C for 5 minutes to ensure RUBISCO activation. To start the assay 50µl 4mM ribulose-1,5-bisphosphate was added and after 5 or 10 minutes the reaction stopped by the addition of 100µl 12M formic acid. Samples (200µl) were evaporated to dryness at 105°C and radioactivity determined as described above.

Phosphoenolpyruvate (PEP) carboxylase (pyrophosphate:oxaloacetate carboxy-lyase (phosphorylating); EC 4.1.1.38) was assayed using cell lysates prepared as for the RUBISCO assay. To 100µl of extract was added 10µl 25mM potassium PEP, 10µl 20mM NADH and 1 unit of malate dehydrogenase. The assay mixture was incubated at 30°C for 10 minutes and the reaction started by the addition of 5µCi NaH¹⁴CO₃. The reaction was stopped after 30 minutes incubation by the addition of 100µl 12M formic acid and 200µl samples treated as described for the RUBISCO assay.

2.6.6) Assay of other enzymes of intermediary metabolism

All enzymes were assayed spectrophotometrically under the same conditions as those employed for TCA cycle enzymes.

The NADH and NADPH oxidase systems were assayed by directly measuring at 340nm the oxidation process. The

reaction mixture contained (μ moles in 3ml):

tris-HCl (pH 7.4)	200.0
NADH or NADPH	0.6

The reaction was started by the addition of cell lysate.

Isocitrate lyase (threo-D₂-isocitrate:glyoxylate lyase; EC 4.1.3.1) was assayed using the method of Reeves et al. (1971). This procedure involves measuring the production of glyoxylate from isocitrate by reacting the latter with phenylhydrazine to produce a hydrazone, the appearance of which is measured at 324nm. The reaction mixture contained (μ moles in 3ml):

tris-HCl (pH 7.5)	100.0
phenylhydrazine-HCl	20.0
L-cysteine	1.0
MgCl ₂	10.0
sodium isocitrate	2.0

The reaction was started by the addition of isocitrate.

Malate synthase (L-malate:glyoxylate lyase (CoA acetylating); EC 4.1.3.2) was assayed at 412nm using a method modified from that used for citrate synthase. The reaction mixture contained (μ moles in 3ml):

tris-HCl	150.0
DTNB	0.6
MgCl ₂	30.0
acetyl-CoA	0.3
sodium glyoxylate	6.0

The reaction was started by the addition of glyoxylate.

2.6.7) Protein assay

This was performed using the method of Lowry et al. (1951) as described by Herbert et al. (1971). To 0.5ml of a suitable dilution of protein sample was added 0.5ml 0.1M NaOH, the sample boiled for 5 minutes to ensure complete solubilisation and rapidly cooled. To 50ml 5% (w/v) Na_2CO_3 was added 2ml of 0.5% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ dissolved in 1% (w/v) sodium potassium tartrate and 2.5ml of this freshly prepared solution added to each protein sample. The samples were incubated at room temperature for 10 minutes and 0.5ml of a 50% (v/v) solution of Folin-Ciocalteu reagent (BDH) rapidly added. The samples were incubated at room temperature for 30 minutes and the A_{750} measured against a blank of double distilled water treated as described above. Actual protein concentration in the samples was determined by reference to a plot of protein concentration against absorbance prepared using standard bovine serum albumin concentrations for each assay. Chromic acid-washed glassware was employed at all times.

2.7) REAGENTS

2.7.1) Chemicals

All standard laboratory reagents employed were of "Analar" standard or the highest purity routinely available and were obtained from the usual laboratory suppliers.

2.7.2) Fine chemicals and biochemicals

Except where specifically stated in the text, all fine chemicals and biochemicals (including enzymes) were obtained from the Sigma (London) Chemical Co.

2.7.3) Radiochemicals

All were obtained from Amersham International plc. All organic acids were supplied as sodium salts. The specific activities of isotopes employed were:

NaH ¹⁴ CO ₃	54.0mCi mmol ⁻¹
[5- ¹⁴ C]-2-oxoglutarate	15.2mCi mmol ⁻¹
[U- ¹⁴ C]-L-malate	60.0mCi mmol ⁻¹
[U- ¹⁴ C]-acetate	<u>c.</u> 55.0mCi mmol ⁻¹
[1- ¹⁴ C]-acetate	<u>c.</u> 55.0mCi mmol ⁻¹
[2- ¹⁴ C]-acetate	<u>c.</u> 55.0mCi mmol ⁻¹

PART 3 - RESULTS AND DISCUSSION

3.1) ECOLOGICAL STUDIES

3.1.1) Observations on natural bacterial populations

Investigations on the bacterial flora of low nutrient fresh water environments was undertaken solely in order to obtain basic qualitative information on the frequency of prosthecate bacteria in such ecosystems. Both Esthwaite Water and the distilled water storage vessels contained a diverse flora which included a variety of morphologically distinctive cells. Although it is naive to classify observed forms into distinct taxa on the basis of observed morphological characteristics, particularly common in both environments were organisms resembling Caulobacter and morphologically similar genera. The morphology of the observed forms was variable (Fig. 3.1). However, the possession of long prosthecae was common and this is a presumed adaptation for increasing surface area to volume ratio and hence improving nutrient uptake efficiency (Poindexter, 1981b). Cells morphologically similar to the Hyphomicrobium group of organisms were also frequently observed in both environments (Fig. 3.2). These normally occurred as chains of cells. More unusual prosthecate morphologies were also observed but no multi-prosthecate forms were seen. Of particular interest was a form morphologically similar to Thiodendron (Fig. 3.2c), although previous observations of this organism have been restricted to sulphur-rich saline waters (Perfilev, 1969; Schmidt, 1980). Non-prosthecate budding bacteria were also observed. Planctomyces (Fig. 3.3a) was only seen in samples

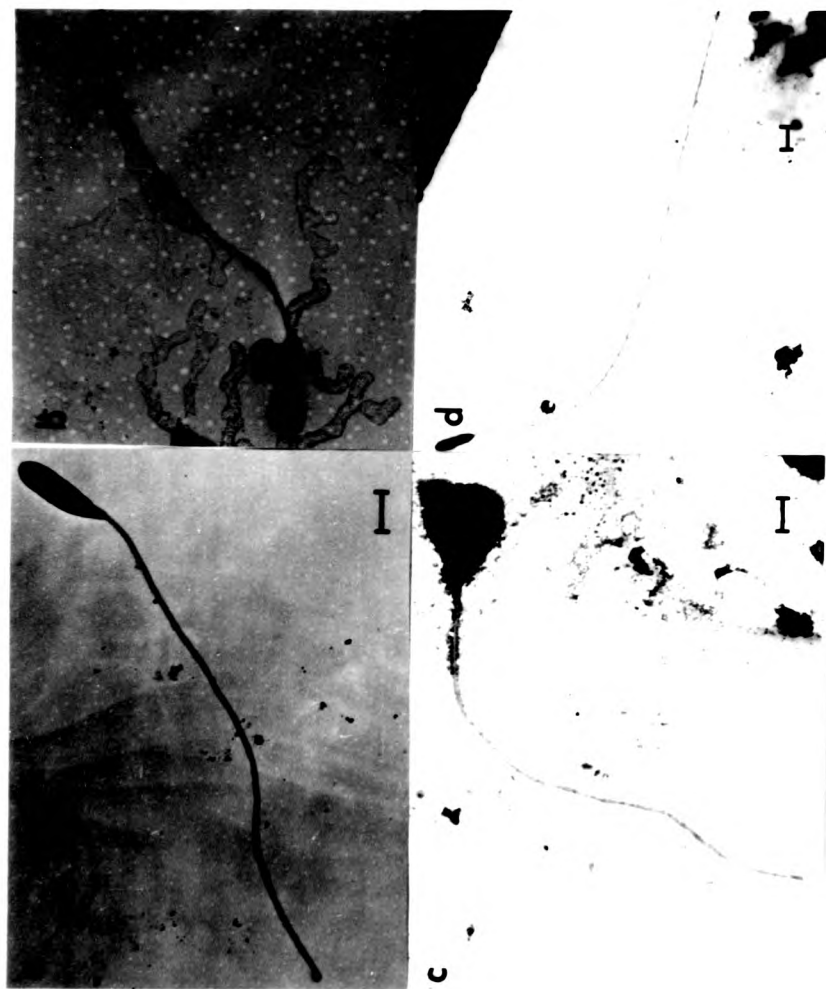


FIGURE 3.1 - *Caulobacter*-like prosthecate bacteria from oligotrophic fresh water environments. (a) Cell observed in double distilled water. (b) Reproducing cell observed in double distilled water. Electron micrographs of gold-palladium shadowed specimens. (c) Cell with angular, elongated main cell body observed in Esthwaite Water. (d) Cell observed in Esthwaite Water. Electron micrographs of phosphotungstic acid stained specimens. Scale bar = 1.0µm.

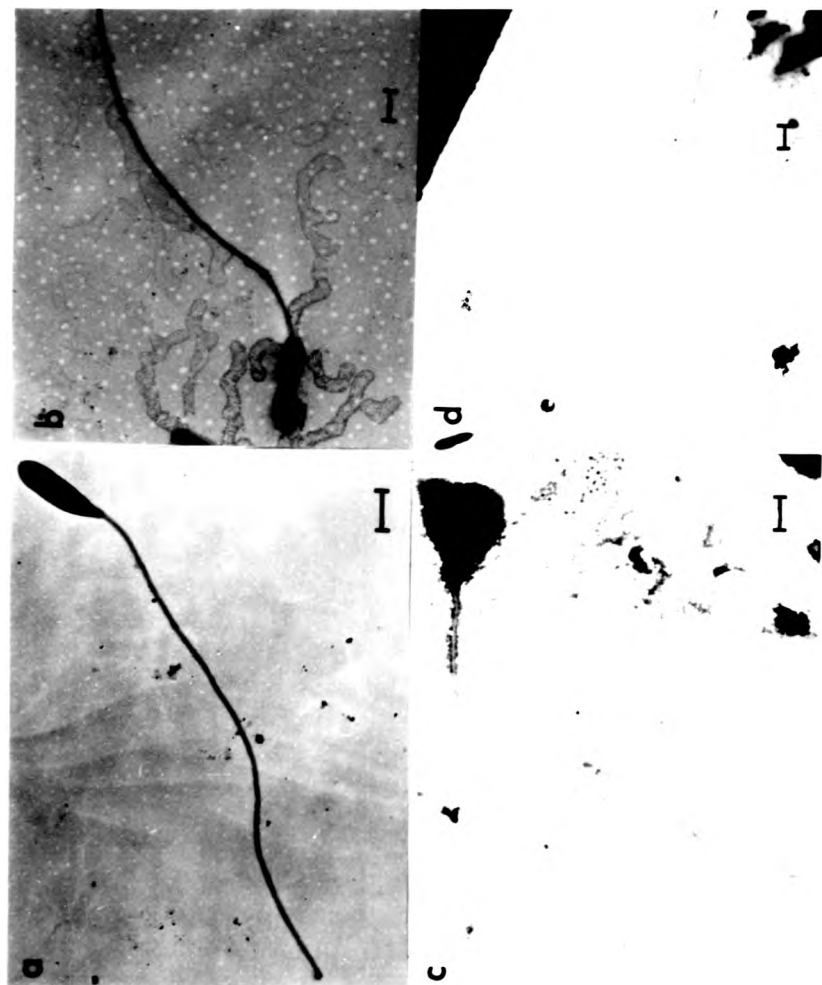


FIGURE 3.1 - Caulobacter-like prosthecate bacteria from oligotrophic fresh water environments. (a) Cell observed in double distilled water. (b) Reproducing cell observed in double distilled water. Electron micrographs of gold-palladium shadowed specimens. (c) Cell with angular, elongated main cell body observed in Esthwaite Water. (d) Cell observed in Esthwaite Water. Electron micrographs of phosphotungstic acid stained specimens. Scale bar = 1.0μm.

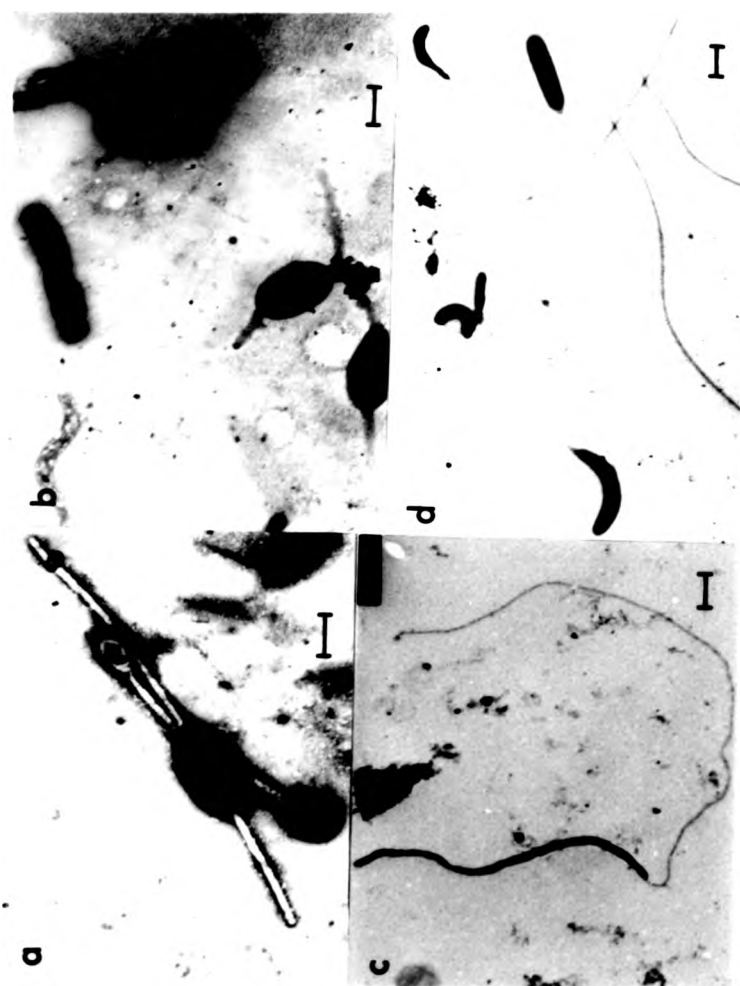


FIGURE 3.2 - Prosthecate bacteria from oligotrophic fresh water environments. (a) and (b) Hyphomicrobium-like cells in association with other non-prosthecate bacteria from Esthwaite Water. Electron micrographs of phosphotungstic acid stained specimens. (c) Prosthecate bacterium, superficially resembling Thiodendron, observed in double distilled water. (d) Caulobacter-like cells in distilled water containing numerous intracytoplasmic inclusions which are most probably storage compounds. Electron micrographs of gold-palladium shadowed specimens. Scale bar = 1.0µm.



FIGURE 3.2 - Prosthecae bacteria from oligotrophic fresh water environments. (a) and (b) Hyphomicrobium-like cells in association with other non-prosthecae bacteria from Esthwaite Water. Electron micrographs of phosphotungstic acid stained specimens. (c) Prosthecae bacterium, superficially resembling Thiodendron, observed in double distilled water. (d) Caulobacter-like cells in distilled water containing numerous intracytoplasmic inclusions which are most probably storage compounds. Electron micrographs of gold-palladium shadowed specimens. Scale bar = 1.0 μ m.

from Esthwaite Water and observed forms were of morphotype III or IV (Schmidt & Starr, 1978). Also of interest in Figure 3.3a is the spherical "umbrella-like" structure. These are commonly observed in studies of oligotrophic ecosystems and are algal scales (Nikitin et al., 1966; Lawrence, 1978). Seliberia were frequently observed in all samples (Fig. 3.3) and occasionally were present in very high numbers. This organism is common in a variety of low nutrient environments (Nikitin & Kuznetsov, 1967; Lawrence, 1978; Schmidt & Swafford, 1981). However, it must be emphasised that, in addition to the morphologically distinct cells, all samples contained high numbers of rods and cocci. A proportion of these forms possessed intracytoplasmic storage granules and some appeared to be budding (Fig. 3.4). In contrast to the observations of Lawrence (1978), where prosthecae bacteria appeared to be largely restricted to the surface areas of lakes, in this study there was no gross discernable variation in the numbers of prosthecae bacteria in samples from various depths of Esthwaite Water. This may have been a function of the sampling time per se (September) or to the turnover of the thermocline.

This study is, by its very nature, subjective and was designed to illustrate the importance and ubiquity of prosthecae bacteria in the oligotrophic fresh water environment. Apart from the absence of multi-prosthecae bacteria, these observations resemble closely those of a number of other workers. Several reports concerning the occurrence of morphologically distinct bacteria in a variety

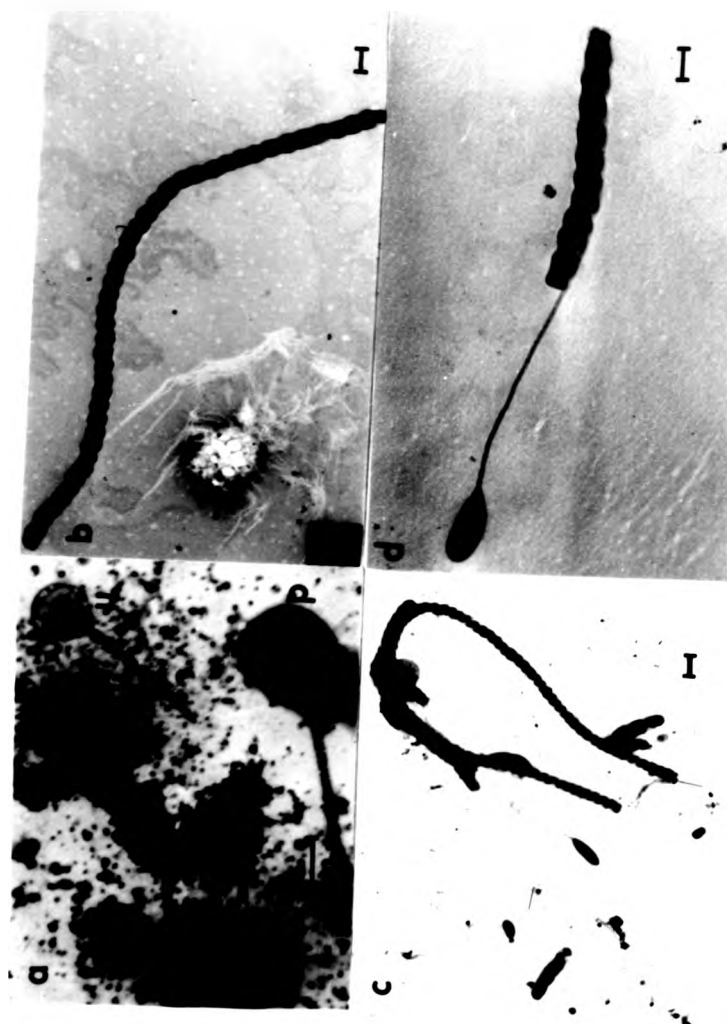


FIGURE 3.3 - Non-prosthecate budding bacteria observed in oligotrophic fresh water environments. (a) Planctomyces species (p) in sediment-rich 16m sample from Esthwaite Water. The spherical "umbrella-shaped" structure (u) is an algal scale (Lawrence, 1978). Electron micrograph of phosphotungstic acid stained specimen. (b) Helically spiral cell of Seliberia in distilled water. (c) Seliberia in association with prosthecate and rod-shaped bacteria in distilled water. (d) Short Seliberia cell in association with Caulobacter. Electron micrographs of gold-palladium shadowed specimens. Scale bar = 1.0µm.



FIGURE 3.3 - Non-prosthecate budding bacteria observed in oligotrophic fresh water environments. (a) Planctomyces species (p) in sediment-rich 16m sample from Esthwaite Water. The spherical "umbrella-shaped" structure (u) is an algal scale (Lawrence, 1978). Electron micrograph of phosphotungstic acid stained specimen. (b) Helically spiral cell of Seliberia in distilled water. (c) Seliberia in association with prosthecate and rod-shaped bacteria in distilled water. (d) Short Seliberia cell in association with Caulobacter. Electron micrographs of gold-palladium shadowed specimens. Scale bar = 1.0μm.

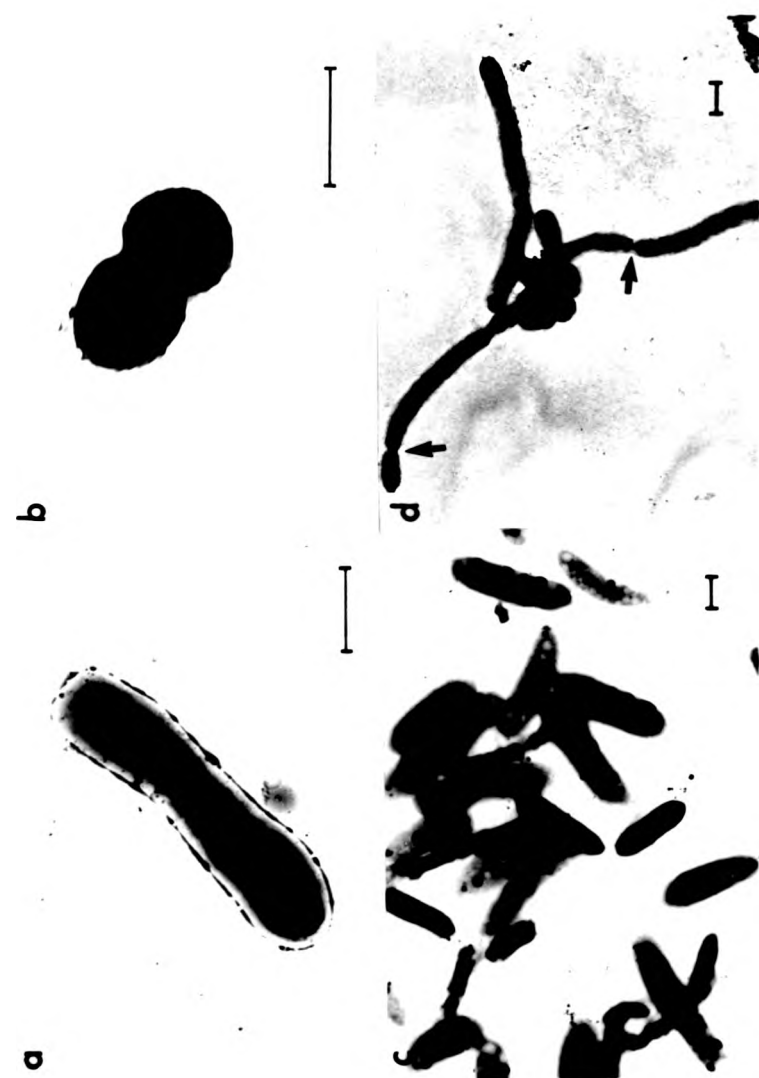


FIGURE 3.4 - Morphologically "typical" bacteria observed in oligotrophic fresh water environments. (a) Short rod, possibly budding, observed in distilled water. (b) Cocci cell from double distilled water. (c) Variety of rod-shaped cells observed in distilled water. Note the large numbers of intracytoplasmic inclusions which are most probably storage compounds. Electron micrographs of gold-palladium shadowed specimens. (d) Long rod, possibly budding, from Esthwaite Water. Note the constriction of the large cells (arrowed) to produce smaller cells. Electron micrograph of phosphotungstic acid stained specimen. Scale bar = 1.0 μ m.

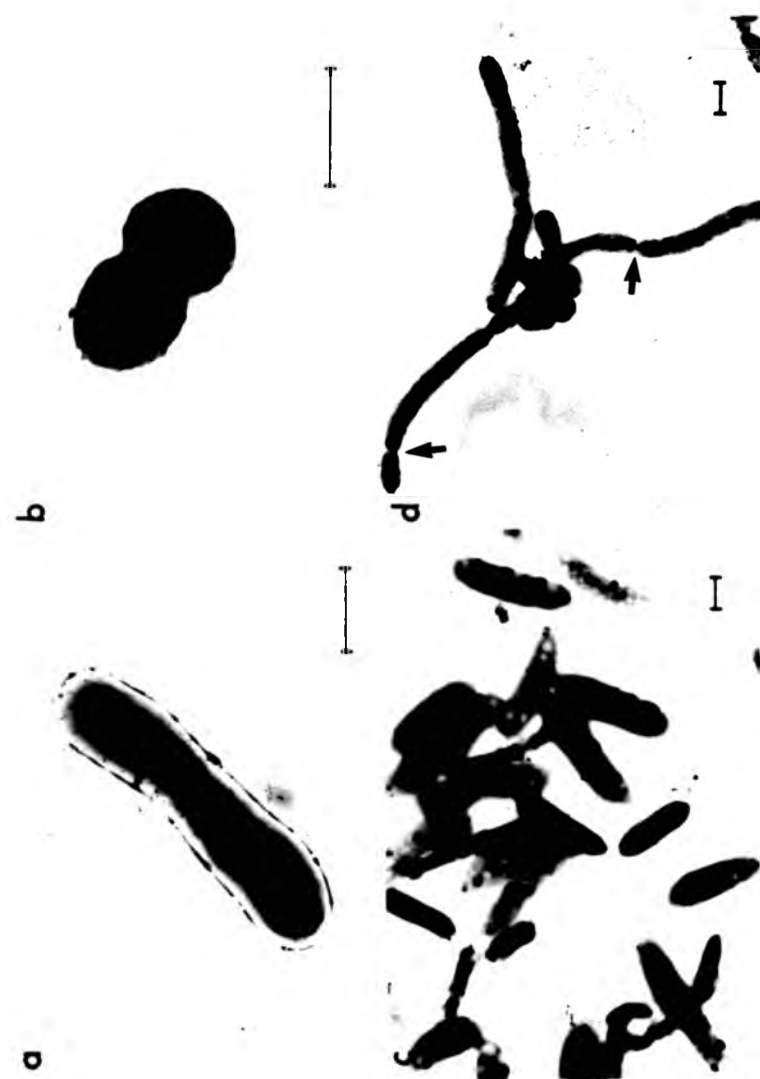


FIGURE 3.4 - Morphologically "typical" bacteria observed in oligotrophic fresh water environments. (a) Short rod, possibly budding, observed in distilled water. (b) Coccoid cell from double distilled water. (c) Variety of rod-shaped cells observed in distilled water. Note the large numbers of intracytoplasmic inclusions which are most probably storage compounds. Electron micrographs of gold-palladium shadowed specimens. (d) Long rod, possibly budding, from Esthwaite Water. Note the constriction of the large cells (arrowed) to produce smaller cells. Electron micrograph of phosphotungstic acid stained specimen. Scale bar = 1.0 μ m.

of oligotrophic ecosystems have been published (e.g. Lawrence, 1978; Dow & Lawrence, 1980; Nikitin & Kuznetsov, 1967; Nikitin et al., 1966) and there are frequent specific reports concerning the occurrence in oligotrophic fresh water of Caulobacter-like (e.g. Callerio et al., 1983; Poindexter, 1981b) and Hyphomicrobium-like (Attwood & Harder, 1972; Hirsch & Conti, 1964) bacteria. Indeed, with notable exceptions, many prosthecae bacteria are restricted to the oligotrophic environment (see Morgan & Dow, 1985). As is confirmed by this brief study these organisms are indeed common in such environments and may represent the dominant proportion of the bacterial flora, however, it must not be forgotten that they coexist with an apparently equally successful morphologically "typical" population (rods and cocci). The proportion of prosthecae and budding bacteria was somewhat higher in the distilled water environment but in both environments there existed a variety of morphologically "typical" cells.

The nutrient sources which are available to the bacteria in the environments studied is open to question. Particularly interesting is the distilled water environment, for whilst the provision of carbon and nitrogen sources by volatiles is to be expected in a laboratory environment, the supply of significant levels of phosphate and other inorganic nutrients is less easy to explain, particularly in vessels used to store freshly prepared double distilled water. It is therefore indicative of the competitive adaptations for scavenging the extremely low levels of nutrients available of

the prosthecate and other indigenous bacteria that there exists a diverse and apparently thriving population under such stringent conditions. The trophic status of Esthwaite Water tends towards the eutrophic which may account for the reduced proportion of prosthecate bacteria observed (Dow & Lawrence, 1980). However, such organisms appear to remain competitive under these conditions in the bulk water. Whilst this study chose to ignore the interface flora in situ, it is probable that in a more detailed study the importance of environmental heterogeneities would be clear and it would be expected that a large number of prosthecate bacteria would be observed at surfaces (e.g. Henrici & Johnson, 1935).

3.1.2) Population development during batch enrichment culture

Samples of double distilled water were incubated with the addition of peptone to a final concentration of 0.01% or 0.001% (w/v), levels frequently used for the enrichment of oligotrophic populations (Poindexter, 1981a). During the initial stages of incubation, the populations in all cultures consisted largely of morphologically "typical" rods with very low numbers of prosthecate cells and Seliberia present. After approximately three months incubation pellicles had formed under both conditions with the predominant bacteria in these being Seliberia and a large rod. The few prosthecate bacteria present were observed in the bulk phase of the water itself. Figure 3.5a illustrates a typical assemblage of bacteria from such a sample with a number of rod-shaped forms

and Seliberia visible. Longer incubation periods of up to one year appeared not to significantly alter the population in 0.01% peptone but in the samples containing 0.001% peptone the proportions of Seliberia in the cultures increased markedly (Fig. 3.5b). Interestingly, although the numbers of prosthecate bacteria in the enrichments did increase with time (Fig. 3.5c), they at no time became the dominant group in the population. Attempts to isolate cells from these enrichments yielded no prosthecate bacteria or Seliberia, however the isolation of both organisms has provided other workers with difficulties (Lawrence, 1978; Schmidt & Swafford, 1981). The bacteria cultivated on agar-solidified media included a variety of rods, cocci and coccobacilli, the majority of which were flagellated and many of which appeared to possess intracytoplasmic nutrient storage polymers.

Samples from Esthwaite Water were incubated without the addition of exogenous nutrients and the population that developed during enrichment was more diverse. After three months incubation the surface samples contained a microflora which included significant numbers of Seliberia, Caulobacter-like cells, Hyphomicrobium-like cells and Planctomyces. The 6m and 10m samples contained rather fewer cells with a smaller proportion of morphologically distinct cells. The 16m samples were enriched in prosthecate and non-prosthecate budding bacteria. Many diatom skeletons and larger cells were visible in these samples. After incubation periods of nine months (Fig. 3.6) the surface samples exhibited a similar mixture of cell types although absolute



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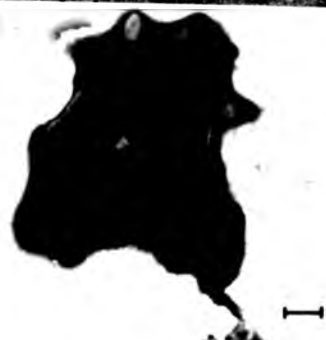


FIGURE 3.5 - Bacterial populations which developed during batch enrichment culture of double distilled water. (a) 3 months incubation at 25°C with the addition of 0.01% (w/v) peptone. Note the large number of ghost cells (examples arrowed) which suggest that many cells have lysed. (b) 6 months incubation at 25°C with the addition of 0.001% (w/v) peptone. (c) 9 months incubation at 25°C with the addition of 0.001% (w/v) peptone. Electron micrographs of gold-palladium shadowed specimens. Scale bar = 1.0µm.



FIGURE 3.5 - Bacterial populations which developed during batch enrichment culture of double distilled water. (a) 3 months incubation at 25°C with the addition of 0.01% (w/v) peptone. Note the large number of ghost cells (examples arrowed) which suggest that many cells have lysed. (b) 6 months incubation at 25°C with the addition of 0.001% (w/v) peptone. (c) 9 months incubation at 25°C with the addition of 0.001% (w/v) peptone. Electron micrographs of gold-palladium shadowed specimens. Scale bar = 1.0µm.

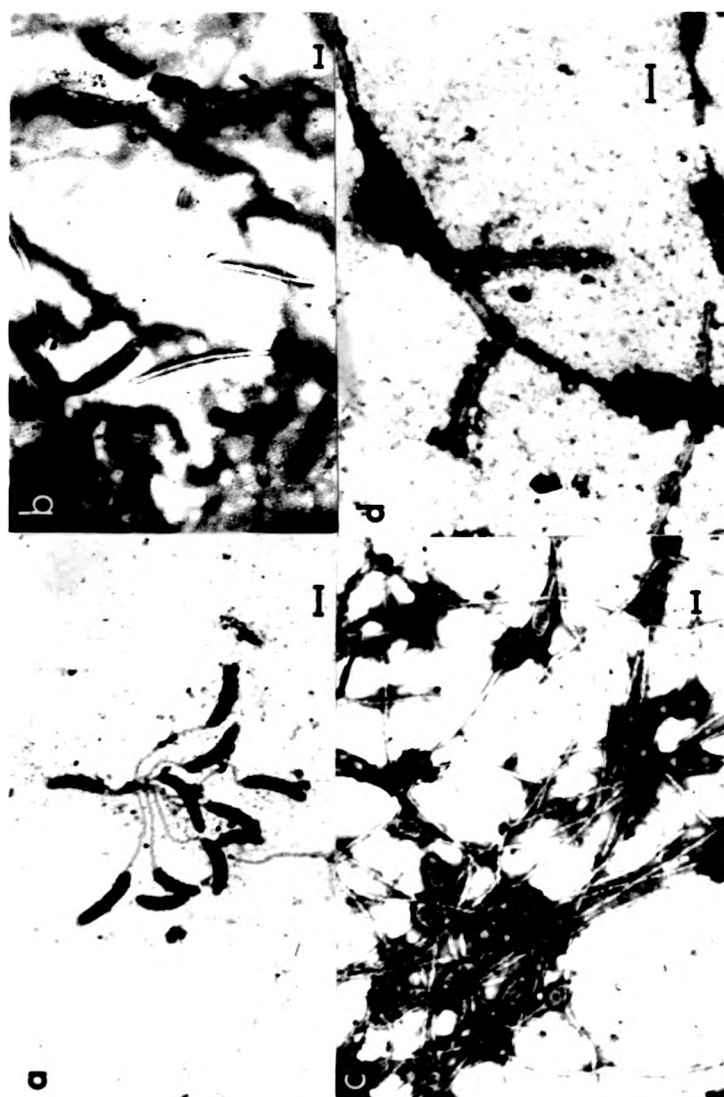


FIGURE 3.6 - Bacterial populations observed in unsupplemented batch enrichment culture of samples from Esthwaite Water. (a) Rosette of Caulobacter-like cells in sample from 6m. (b) Seliberia and rod-shaped cells in sample from 10m. (c) and (d) Hyphomicrobium-like cells in sample from 16m. Electron micrographs of phosphotungstic acid stained specimens. Scale bar = 1.0 μ m.

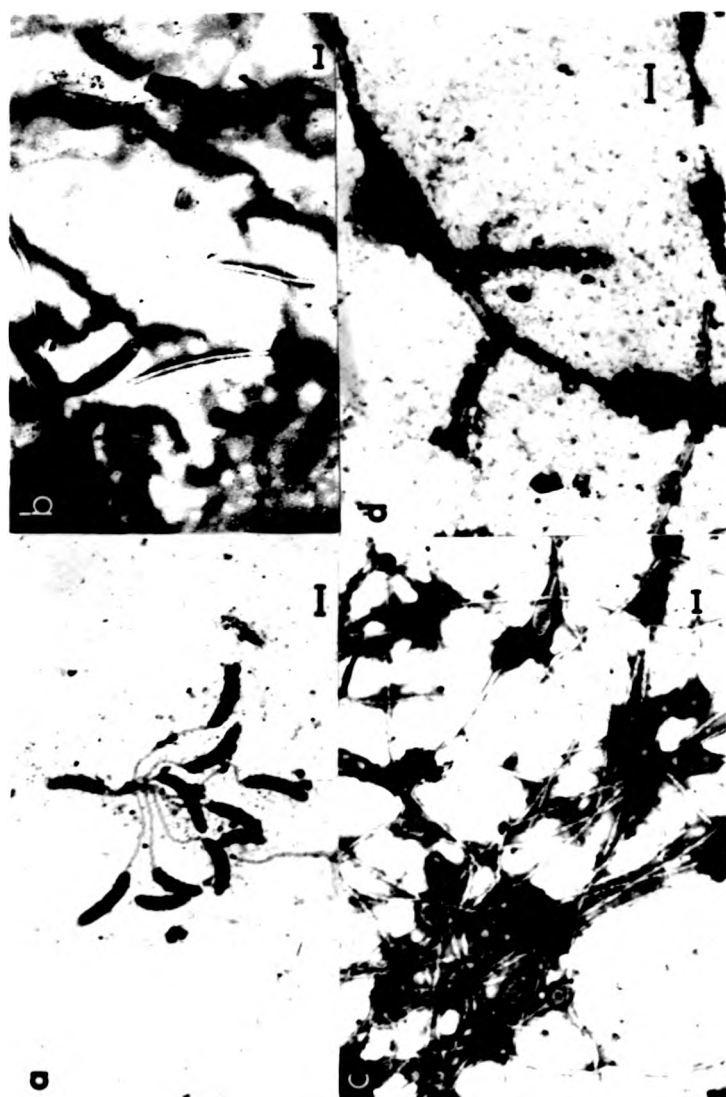


FIGURE 3.6 - Bacterial populations observed in unsupplemented batch enrichment culture of samples from Esthwaite Water. (a) Rosette of Caulobacter-like cells in sample from 6m. (b) Seliberia and rod-shaped cells in sample from 10m. (c) and (d) Hyphomicrobium-like cells in sample from 16m. Electron micrographs of phosphotungstic acid stained specimens. Scale bar = 1.0µm.

numbers appeared to have declined, suggesting that the initial population size may have been due to nutrients leaking from either whole or lysing cells originally present in the surface layer of the lake. The samples from deeper in the lake contained populations similar to those of three months with an increased frequency of prosthecate bacteria observed. In contrast to previous observations (Lawrence, 1978), no differences in the proportions of prosthecate bacteria through the depth profile were apparent. Once again, all cells isolated from these enrichments were morphologically "typical". In the light of observations that some bacteria from oligotrophic environments tend to lyse on contact with solid surfaces out of the aquatic environment (Fig. 3.5a; C.S. Dow, unpublished observations), it is possible that this difficulty in isolation is a function of mechanical disruption of fragile cells on an agar surface rather than a nutritional effect.

These observations differ from those made by others.

Lawrence (1978)

observed a rapid decline in the numbers of rod-shaped and coccoid cells and concomitant increases in the numbers of prosthecate and non-prosthecate budding forms during static batch enrichment of samples from Draycote Water, Warwickshire and Derwent Water, Lake District. These data were taken to be indicative of the prosthecate bacteria being specialised oligotrophs. The absence of multi-prosthecate cells from the described enrichments is also in contradiction to previous observations where these organisms became common

with increasing incubation periods. The large numbers of Seliberia during this type of enrichment culture has previously been noted (Schmidt & Swafford, 1981), although Lawrence (1978) did not report significant numbers of these bacteria in her enrichments. These discrepancies may reflect differences in the nutrient levels of the environments sampled.

This study, of course, yields no information on the relative activities of the bacteria in the enrichment cultures. It is clear, both from the relative numbers of cells in the enrichments and the populations developing on agar-solidified media, that they remain viable and competitive in situ, although it is possible that a proportion of the cells may well be dormant. It must be emphasised that it is not unusual for morphologically "typical" cells to be successful in the oligotrophic environment. Many of the described forms of oligocarbophilic bacteria isolated from the natural environment have been classified as belonging to common genera (Hirsch, 1964; Witzel et al., 1982). Similarly, many of the isolated "oligotrophic" bacteria are morphologically unspecialised and can competitively co-exist with prosthecae bacteria in situ (e.g. Akagi et al., 1977; Mallory et al., 1977; Austin et al., 1979).

The long prosthecae observed in these enrichments are indicative of extreme nutrient limitation on the cells (Poindexter, 1981b). The nutrient sources available to the cells in the unsupplemented enrichment cultures were unknown, for whilst cellular products would have been excreted into the medium, for whatever reason, it is possible that

atmospheric volatiles were functioning at least in part as nutrient sources. In those cultures supplemented with peptone it may have been a consequence of the relatively high concentration of nutrients that a predominantly morphologically "typical" population developed. However, Esthwaite Water is somewhat eutrophic (Dow & Lawrence, 1980) yet supports a diverse and successful prosthecate and non-prosthecate budding bacterial flora. In the light of these observations it is clear that the allocation of the prosthecate bacteria to a distinct group of "model oligotrophs" is by no means as simple as has been proposed (Hirsch, 1979; Poindexter, 1981a; 1981b). The rapid responses of the "typical" cells to increased concentrations of nutrients on plating is also of interest, since it illustrates that competitive success in an oligotrophic environment requires the ability to respond rapidly to nutrients as they become available. It would be most enlightening to study the relative activities of the different bacterial forms under both nutrient stress and on nutrient enrichment.

From these basic observations two major conclusions can be drawn. Firstly, whilst it must be emphasised that a proportion of the prosthecate population will be unrecognised since they will be present as morphologically indistinctive swarmer cells, it is clear that prosthecate and non-prosthecate budding bacteria are by no means the only successful forms in the low nutrient environment. Secondly, the absence of prosthecate bacteria in populations developing

following subculture onto agar-solidified media suggests that these organisms may be less able to respond to nutrient enrichment and may be consequently less competitive in those oligotrophic environments subject to periodical major nutrient influx.

3.1.3) Population development during continuous flow enrichment culture

In contrast to batch enrichment culture, the continuous flow enrichment culture technique employed in this study made some attempt to incorporate some of the features of the environment from which the samples were taken. The distilled water storage systems sampled contained some surfaces and were subject to continuous inflow and removal of "medium". Both of these were in some way incorporated into this technique, although both the surface area available on the glass beads and the concentration of nutrients in the media employed were somewhat higher than those in the original system. Whilst this would undoubtedly have affected the populations observed, the media employed are typical of those employed in studies on oligotrophic bacteria and were chosen to provide a means of reference to published observations.

The 0.01% (w/v) Difco Bacto-peptone medium supplies approximately 42mg organic carbon l^{-1} (Anon., 1953; Martin & MacLeod, 1984), a carbon concentration which is somewhat above that considered inhibitory to obligate oligotrophs (Kuznetsov *et al.*, 1979) but one that is characteristically employed in studies of oligotrophic

bacteria. This medium was employed at flow rates of between 0.16 and 0.49ml min⁻¹. Cell density and diversity in the bulk medium was observed to increase with increasing flow rate but under all conditions the greatest apparent cell density was observed at surfaces. At low flow rates Seliberia and prosthecate cells were observed in significant numbers, the latter particularly at surfaces, but these were absent at high flow rates. Figure 3.7 illustrates the populations developing at three flow rates. At 0.29ml min⁻¹ (Figs. 3.7a,b) the bulk medium consisted of a sparse population of rod-shaped cells and a very few prosthecate cells, whereas at interfaces a dense population including large numbers of prosthecate cells was observed. At 0.38ml min⁻¹ (Fig. 3.7c,d) the population in the medium was denser and consisted of a variety of rods and a very few prosthecate cells and at interfaces the relative numbers of prosthecate cells was decreased. At a flow rate of 0.49ml min⁻¹ (Fig. 3.7e,f) both medium and surface populations lacked prosthecate cells but a variety of morphologically "typical" forms were observed many of which contained intracellular nutrient storage compounds.

The minimal medium (Staley, 1981a) with 0.002% glucose was employed at flow rates of between 0.1 and 1.1ml min⁻¹ but despite the lower concentration of organic carbon appeared to support a morphologically more diverse population of bacteria and permitted the growth of fungi which were rarely observed in peptone cultures. Once again at low flow rates the populations supported were primarily surface-associated and

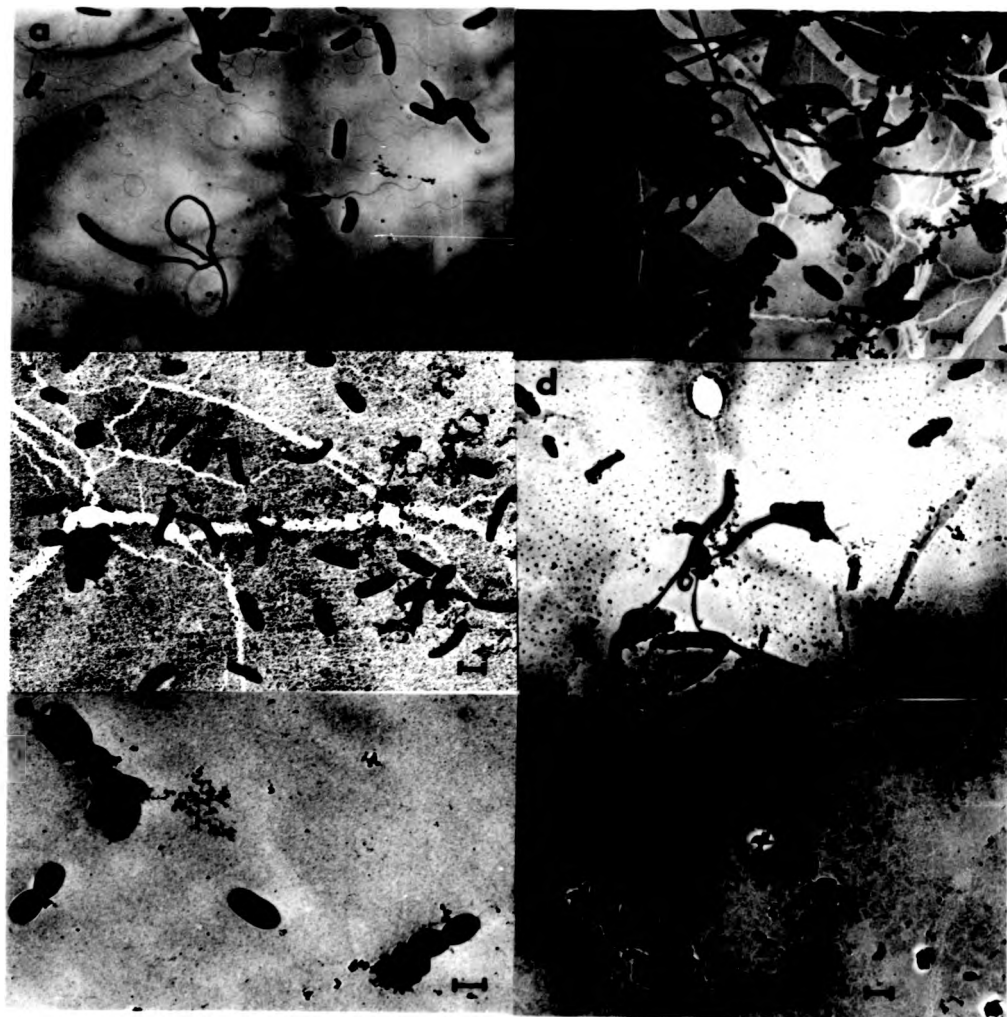


FIGURE 3.7 - Populations which developed during continuous flow enrichment culture of oligotrophic bacteria using 0.01% (w/v) peptone medium. (a) Bulk medium, flow rate = 0.29ml min⁻¹. Note the large numbers of flagella in this preparation. (b) Glass surface, flow rate = 0.29ml min⁻¹. (c) Bulk medium, flow rate = 0.38ml min⁻¹. (d) Glass surface, flow rate = 0.38ml min⁻¹. (e) Bulk medium, flow rate = 0.49ml min⁻¹. (f) Glass surface, flow rate = 0.49ml min⁻¹. Electron micrographs of gold-palladium shadowed specimens. Scale bar = 1.0μm.

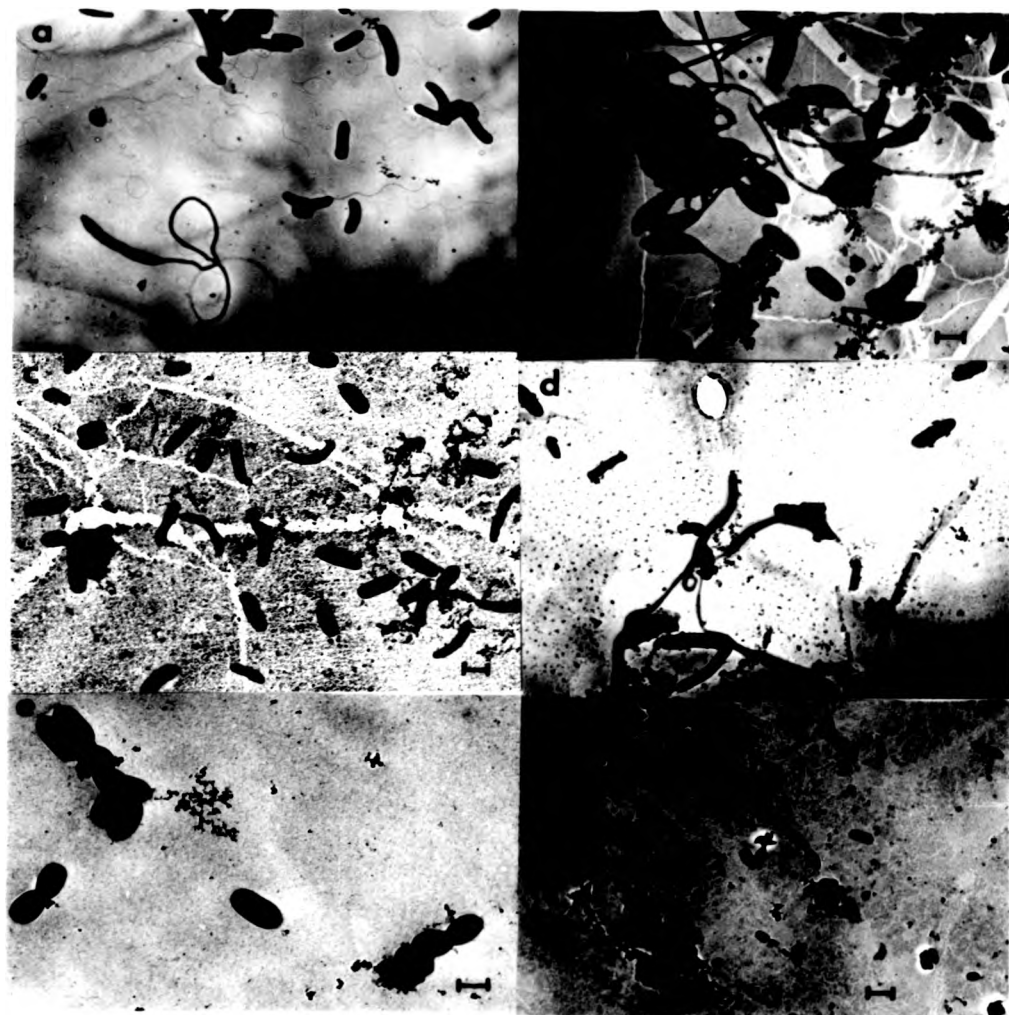


FIGURE 3.7 - Populations which developed during continuous flow enrichment culture of oligotrophic bacteria using 0.01% (w/v) peptone medium. (a) Bulk medium, flow rate = 0.29ml min⁻¹. Note the large numbers of flagella in this preparation. (b) Glass surface, flow rate = 0.29ml min⁻¹. (c) Bulk medium, flow rate = 0.38ml min⁻¹. (d) Glass surface, flow rate = 0.38ml min⁻¹. (e) Bulk medium, flow rate = 0.49ml min⁻¹. (f) Glass surface, flow rate = 0.49ml min⁻¹. Electron micrographs of gold-palladium shadowed specimens. Scale bar = 1.0μm.

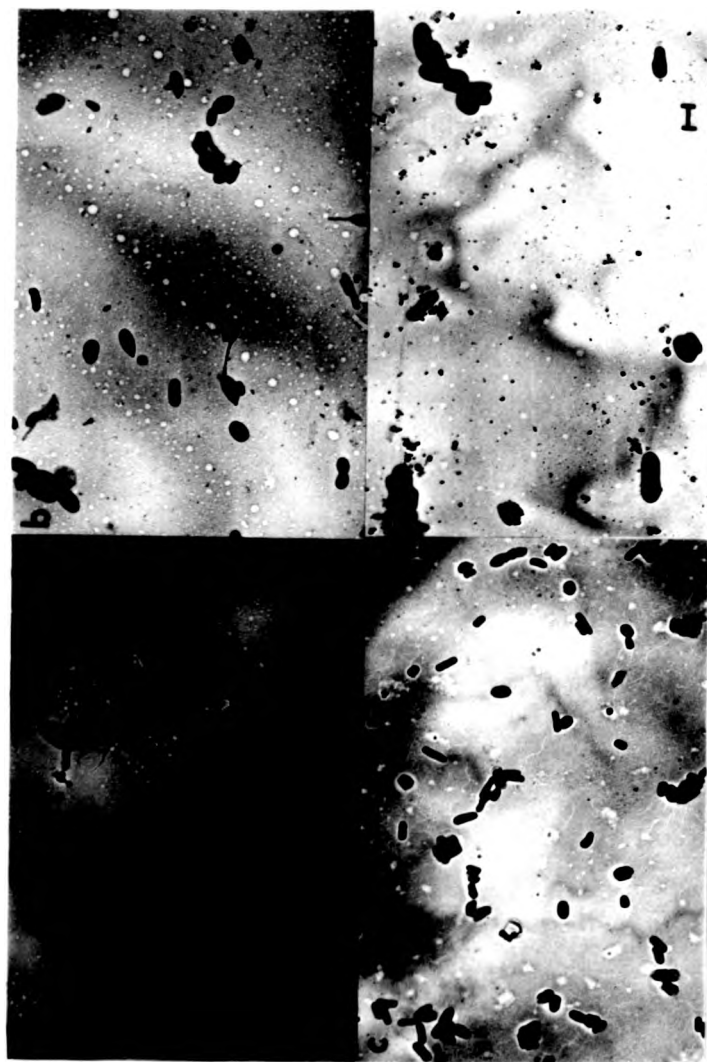


FIGURE 3.8 - Populations which developed during continuous flow enrichment culture of oligotrophic bacteria using minimal medium. (a) Bulk medium, flow rate = 0.12 ml min^{-1} . (b) Glass surface, flow rate = 0.12 ml min^{-1} . (c) Bulk medium, flow rate = 0.40 ml min^{-1} . (d) Glass surface, flow rate = 0.40 ml min^{-1} . Medium of Staley (1981a) containing 0.002% (w/v) glucose. Electron micrographs of gold-palladium shadowed specimens. Scale bar = $1.0 \mu\text{m}$.

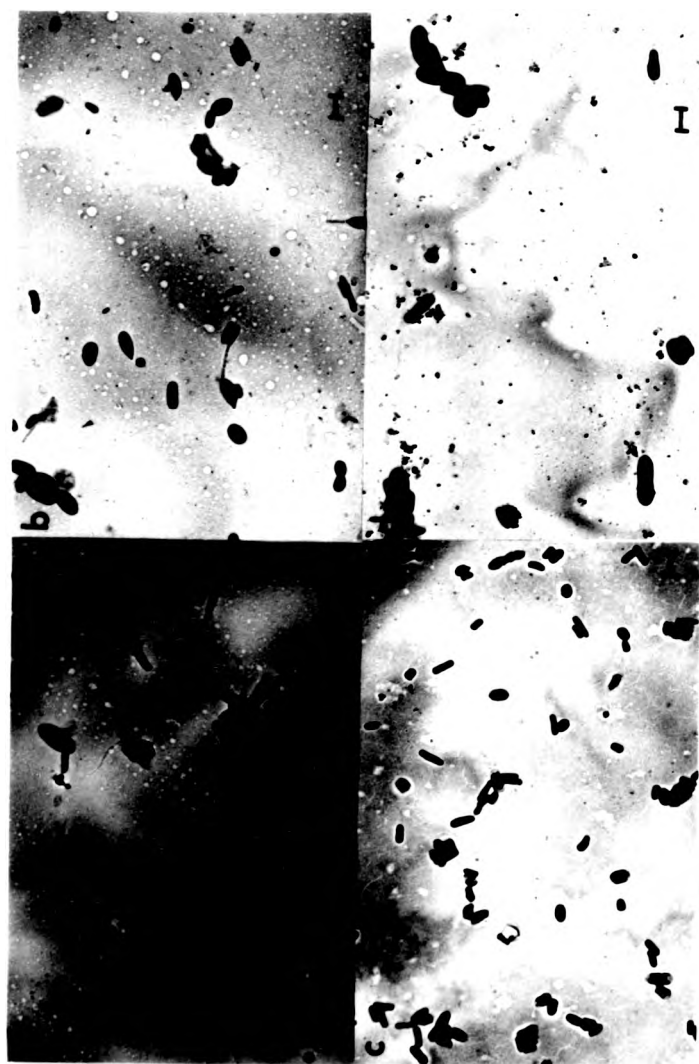


FIGURE 3.8 - Populations which developed during continuous flow enrichment culture of oligotrophic bacteria using minimal medium. (a) Bulk medium, flow rate = 0.12ml min⁻¹. (b) Glass surface, flow rate = 0.12ml min⁻¹. (c) Bulk medium, flow rate = 0.40ml min⁻¹. (d) Glass surface, flow rate = 0.40ml min⁻¹. Medium of Staley (1981a) containing 0.002% (w/v) glucose. Electron micrographs of gold-palladium shadowed specimens. Scale bar = 1.0μm.

included prosthecate bacteria in relatively high numbers. With increasing flow rates these were less common in the population and the flora observed consisted of a variety of morphologically "typical" cells, many of which contained storage compounds within the cells. Figure 3.8 illustrates the populations which developed at two flow rates. At 0.12 ml min^{-1} (Fig. 3.8a,b) the sparse bulk medium population consisted of a variety of small rod-shaped cells and at the surfaces prosthecate cells were observed. At a flow rate of 0.40 ml min^{-1} (Fig. 3.8c,d) both populations consisted of a diverse variety of rod-shaped cells but prosthecate cells were virtually absent.

Samples from all experiments were streaked onto agar-solidified versions of the media employed and incubated at 25°C for four weeks. In general, the populations developing were morphologically similar to those in the samples themselves but in a number of cases there was an increase in the observed frequency of prosthecate bacteria, particularly in samples of the bulk medium (Fig. 3.9). Whilst this may merely reflect differences in the competitiveness of the organisms under the different cultural conditions, it is possible that this may also indicate an underestimation of the numbers of prosthecate bacteria by virtue of the existence of swarmer cells which are morphologically indistinct from the bulk of the population. Dow *et al.* (1983) proposed that the swarmer cells of prosthecate bacteria represented specialised motile survival and dispersal stages that would only initiate

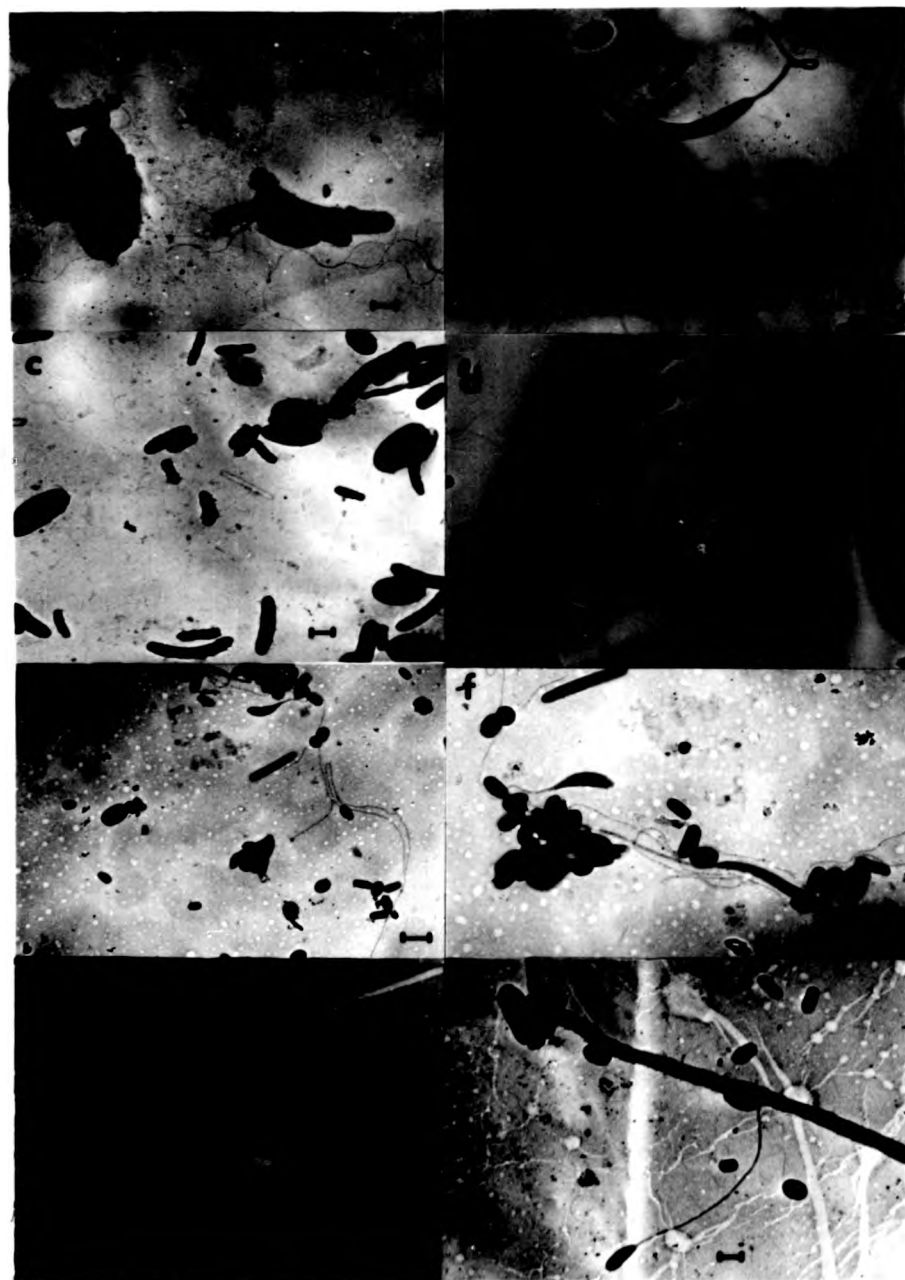


FIGURE 3.9 - Populations from continuous flow enrichment culture which developed on agar-solidified versions of the culture media. (a) Bulk medium, flow rate = 0.38 ml min^{-1} . (b) Glass surface, flow rate = 0.38 ml min^{-1} . (c) Bulk medium, flow rate = 0.38 ml min^{-1} . (d) Glass surface, flow rate = 0.38 ml min^{-1} . 0.01% (w/v) peptone-agar plates incubated at 25°C for 4 weeks. (e) Bulk medium, flow rate = 0.12 ml min^{-1} . (f) Glass surface, flow rate = 0.12 ml min^{-1} . (g) Bulk medium, flow rate = 0.40 ml min^{-1} . (h) Glass surface, flow rate = 0.40 ml min^{-1} . Plates of agar-solidified minimal medium (Staley, 1981a) with 0.002% (w/v) glucose incubated at 25°C for 4 weeks. Electron micrographs of gold-palladium shadowed specimens. Scale bar = $1.0 \mu\text{m}$.

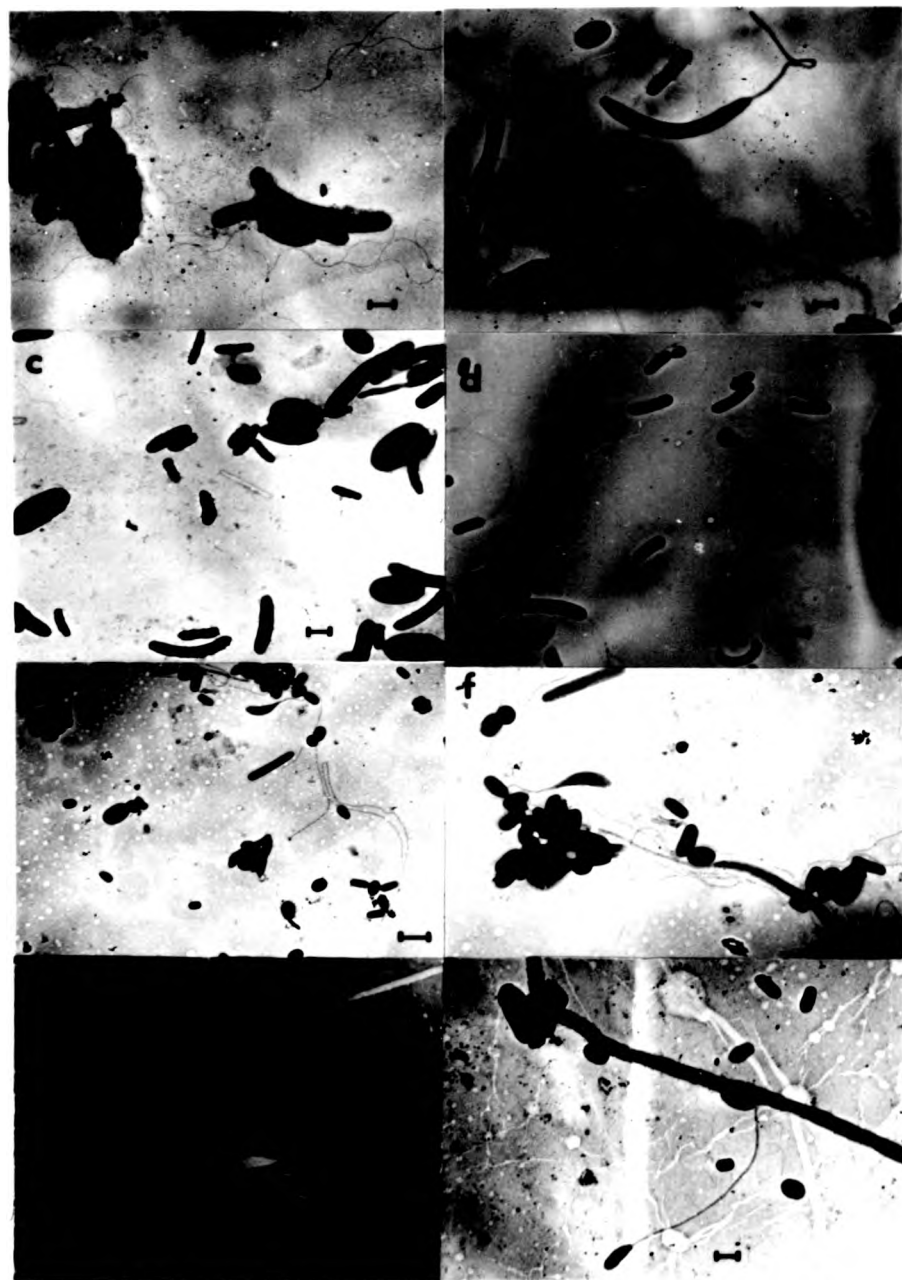


FIGURE 3.9 - Populations from continuous flow enrichment culture which developed on agar-solidified versions of the culture media. (a) Bulk medium, flow rate = 0.38ml min^{-1} . (b) Glass surface, flow rate = 0.38ml min^{-1} . (c) Bulk medium, flow rate = 0.38ml min^{-1} . (d) Glass surface, flow rate = 0.38ml min^{-1} . 0.01% (w/v) peptone-agar plates incubated at 25°C for 4 weeks. (e) Bulk medium, flow rate = 0.12ml min^{-1} . (f) Glass surface, flow rate = 0.12ml min^{-1} . (g) Bulk medium, flow rate = 0.40ml min^{-1} . (h) Glass surface, flow rate = 0.40ml min^{-1} . Plates of agar-solidified minimal medium (Staley, 1981a) with 0.002% (w/v) glucose incubated at 25°C for 4 weeks. Electron micrographs of gold-palladium shadowed specimens. Scale bar = $1.0\mu\text{m}$.

differentiation when environmental conditions were suitable for growth. In the light of this suggestion it is possible that the relative rarity of prosthecate bacteria in the bulk medium samples, particularly at higher medium flow rates, was a reflection of this function. Prosthecate cells at interfaces produce swarmer cells which are released into the medium where they do not differentiate until they encounter a suitable niche. If, as a consequence of competition in the bulk phase of the enrichments few underwent differentiation, it would be expected that the majority of prosthecate cells would be observed at interfaces where localised concentration of nutrients would occur. However, it must be emphasised that nothing is known regarding the triggering of differentiation in the swarmer cells of chemotrophic prosthecate bacteria and it is possible that interspecific competition or a variety of physical factors may also be responsible for the observed effects.

In excess of 90 bacterial isolates were obtained from these experiments of which only two were prosthecate bacteria. Despite the ubiquity of both the prosthecate bacteria and Seliberia in certain samples, these organisms once again proved difficult to isolate in pure culture. The vast majority of the rod-shaped bacteria, cocci and coccobacilli isolated were actively motile and many were capable of accumulating nutrient storage compounds, both adaptations believed to be important in ensuring survival and competitiveness in the natural low nutrient environment (Hirsch, 1979; Morgan & Dow, 1986). The two prosthecate

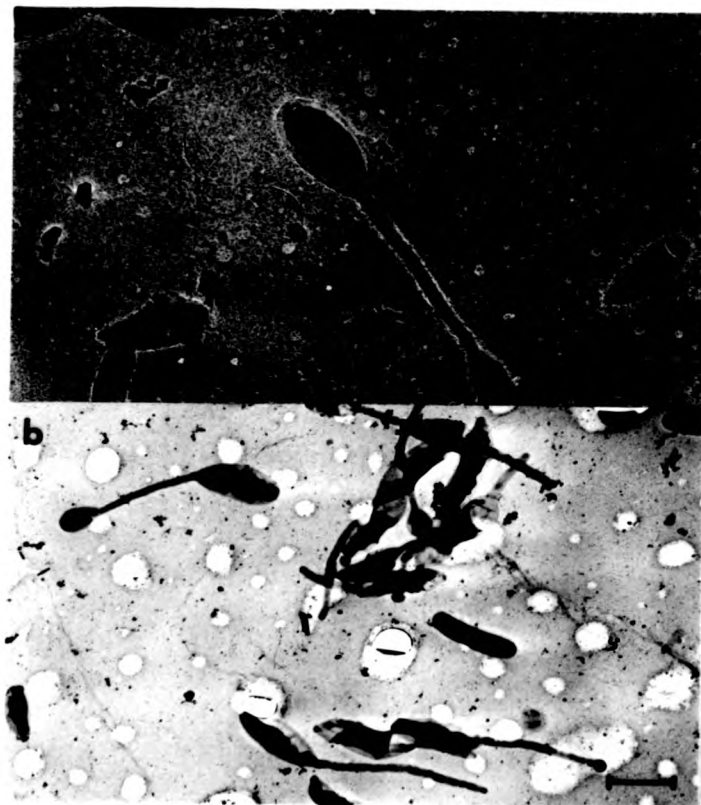


FIGURE 3.10 - *Hyphomonas*-like bacteria isolated from continuous flow enrichment culture. (a) Isolate 9/82 from bulk medium at a flow rate of 0.12ml min^{-1} . (b) Isolate 8/wg1 from glass surfaces at a flow rate of 0.43ml min^{-1} . Minimal medium (Staley, 1981a) containing 0.002% (w/v) glucose. Electron micrographs of gold-palladium shadowed specimens. Scale bar = $1.0\mu\text{m}$.

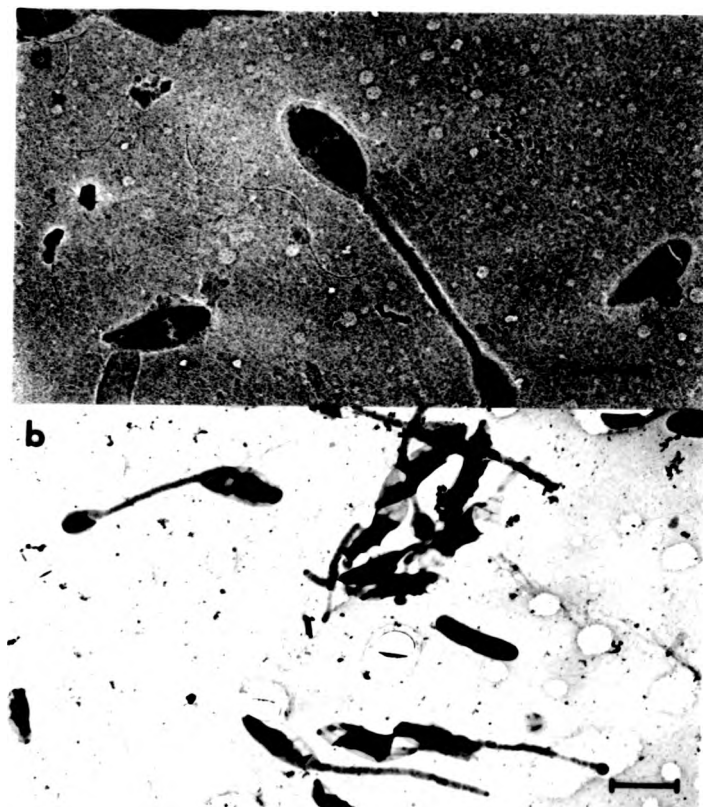


FIGURE 3.10 - *Hyphomonas*-like bacteria isolated from continuous flow enrichment culture. (a) Isolate 9/S2 from bulk medium at a flow rate of 0.12ml min^{-1} . (b) Isolate B/wg1 from glass surfaces at a flow rate of 0.43ml min^{-1} . Minimal medium (Staley, 1981a) containing 0.002% (w/v) glucose. Electron micrographs of gold-palladium shadowed specimens. Scale bar = $1.0\mu\text{m}$.

strains isolated (Fig. 3.10) were morphologically similar to Hyphomicrobium in that reproduction occurred by the production of a bud at the distal end of the prostheca and on account of their ability to use glucose as a carbon source were most probably members of the genus Hyphomonas (Moore et al., 1984).

These observations serve not only to demonstrate that prosthecate bacteria are successful in the low nutrient environment but that under all conditions they coexist with a variety of apparently equally competitive morphologically "typical" cells. The physiological states of the organisms in the environments examined, in particular whether they are quiescent, growing or actively reproducing, remains unclear. It is, however, evident that the prosthecate bacteria in this study were most common when nutrient conditions were the most severe and were proportionally less frequent as the nutrient flow rates increased and on subculture to solid media. This does not demonstrate that the prosthecate bacteria are inhibited by increased nutrient concentrations per se but that they are inactivated on subculture or are uncompetitive with respect to other members of the population. This study does not, for example, provide any information regarding changes in the morphologically "typical" bacterial population which may be equally noteworthy in terms of the "trophic" nature of the ecosystem. It is clear that in addition to the morphologically distinctive "model" oligotrophs (Poindexter, 1981a), many of the indigenous and successful bacteria of low nutrient environments are morphologically "ordinary" (e.g.

Akagi et al., 1980; Mallory et al., 1977).

Thus the prosthecate bacteria are by no means the only successful bacteria in the fresh water low nutrient environment but appear to be somewhat specific for more limiting conditions in a natural mixed population. It is of interest to note that under more limiting conditions the major portion of the bacterial flora was observed at solid-liquid interfaces where localised concentration of nutrients occurs (Fletcher & Marshall, 1982) and that few cells were free-floating. Observations on the latter population on solid media suggested that at least a proportion of the cells may have been swarmer cells of prosthecate bacteria. Therefore, although it must be emphasised that there exist a variety of cells capable of growth in the low nutrient environment, it can be proposed that the prosthecate bacteria may represent organisms which are visibly specialised organisms for oligotrophic existence.

3.1.4) The effects of nutrient concentration on natural isolates

Selected isolates from continuous flow enrichment cultures were cultivated in minimal medium containing various concentrations of glucose in order to investigate their responses to increased levels of organic carbon. For comparison, E. coli C and C. crescentus CB15 were also investigated as a supposed model copiotroph and oligotroph respectively (Poindexter, 1981a; 1981b). The growth yields in batch culture were measured by A₄₀₀ and the results are

TABLE 3.1 - Growth yields of oligotrophic isolates as a consequence of glucose concentration. Growth yield (A_{400}) was measured at stationary phase following aerobic incubation at 25°C of *E. coli* C, *C. crescentus* CB15 and isolates from continuous flow enrichment cultures using 0.01% (w/v) peptone.

Isolate	Isolation flow rate (ml min ⁻¹)	Source	<u>A_{400} at early stationary phase at glucose concentrations of (mg l⁻¹);</u>			
			10	100	1000	10000
<i>E. coli</i>	---	-	.015	.145	.405	.839
CB15	---	-	.008	.155	.380	.800
2/gb1	.17	S ^a	.015	.182	.115	.110
2/wg4	.17	S	.008	.155	.258	.249
1/wg3	.36	S	.000	.105	.279 ^b	.234 ^b
1/wf1	.36	S	.020	.200	.205	.239
3/wg2	.38	S	.005	.140	.430	.440 ^b
3/wg3	.38	S	.005	.155	.330	.235
4/P2	.49	S	.010	.124	.250	.240 ^b
4/gb5	.49	S	.010	.200	.210	.240
2/S2	.17	B ^a	.008	.145	.189	.059
1/S5	.36	B	.050	.175	.685	.670
3/S2	.38	B	.005	.110	.174	.148
3/S3	.38	B	.010	.142	.230	.118
4/S3	.49	B	.015	.145	.260	.780 ^b

(a) S, surface; B, bulk medium.

(b) Flocculent growth, A_{400} measurements therefore underestimate yield.

presented in Table 3.1. Two distinct types of responses were observed. Both *E. coli* and *C. crescentus*, along with some of the isolates (for example 3/wg2), displayed a yield that was continually proportional to the concentration of glucose over the range tested, i.e. they displayed a typically

copiotrophic response. However, certain isolates (for example 2/gb1) were apparently inhibited by the highest nutrient concentrations, a response characteristically described as oligotrophic and similar to a number of previous reports (e.g. Akagi et al., 1980; Ishida & Kadota, 1981; Yanagita et al., 1978). Interestingly, no isolates from the highest flow rates in the enrichment culture displayed the latter response. Furthermore, there was no apparent difference in the distribution of the two types of response between bacteria originating from the bulk medium and those from surfaces. To further illustrate these responses isolates 3/wg2 and 2/gb1 were cultivated in the same way using a larger number of glucose concentrations. The results (Fig. 3.11) confirmed the above observations.

The use of 0.01% peptone as the isolation medium is characteristic of many studies involving the isolation of oligotrophic bacteria which either use this carbon source or another complex mixture of nutrients (reviewed in Kuznetsov et al., 1979; Poindexter, 1981a). However, some workers have stated that their "obligate" oligotrophs from the natural ecosystem isolated by such means were incapable of using glucose as a carbon source and were solely associated with surfaces (Ishida et al., 1980; Ishida & Kadota, 1981). The described results contradict such observations in both respects, of the 20 bacteria isolated on peptone tested, only two were incapable of utilising glucose and there was an apparently equal distribution of organisms displaying nutrient sensitivity among isolates from surfaces and the

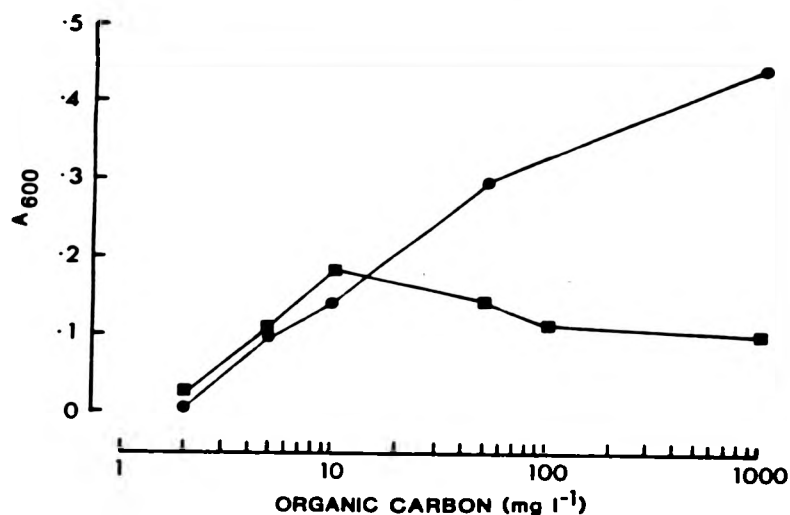


FIGURE 3.11 - The two types of responses of oligotrophic isolates to varying glucose concentrations. Measurements of A_{600} were used to determine yields of bacteria in the early stationary phase of growth in minimal medium (Staley, 1981a) containing variable concentrations of glucose. Isolate 3/wg2 (\bullet) exhibits a direct proportionality between organic carbon and growth yield in batch culture. Isolate 2/gb1 (\blacksquare) had a maximal yield at a relatively low organic carbon concentration and was inhibited at higher concentrations.

bulk medium. The behaviour of C. crescentus and E. coli in these studies was anomalous. The former organism did not display any nutrient sensitivity in this system and the latter was able to grow successfully in "oligotrophic" media.

To provide more detailed information on the responses to nutrient levels of the "model" organisms and the naturally isolated potentially oligotrophic prosthecate bacteria, E. coli C, C. crescentus CB15 and the prosthecate isolates 9/S2 and B/wg1⁸ were investigated using a variety of carbon sources. In view of the common use of peptone in studies of oligotrophic bacteria, these organisms were initially tested for their responses to increasing levels of this product. The results are illustrated in Figure 3.12. Both E. coli and C. crescentus responded similarly over the range of concentrations tested which, as discussed above, suggests that these organisms do not fit into their model trophic groupings. The response of isolate 9/S2 (Fig. 3.12c) shows a clear maximum yield at approximately 500mg organic carbon l⁻¹. Whilst this value is very much higher than that proposed as an optimum carbon concentration for oligotrophic bacteria (Kuznetsov et al., 1979), the inhibition of growth at higher concentrations of organic carbon is characteristic of natural "oligotrophic" isolates (Ishida & Kadota, 1981; Hattori & Hattori, 1980; Akagi et al., 1980). The response of isolate B/wg1 (Fig. 3.12d) appears to be of a somewhat intermediate nature with no marked increase or decrease in growth yield apparent at higher concentrations. It is interesting to note that, at the

see Fig. 3.10

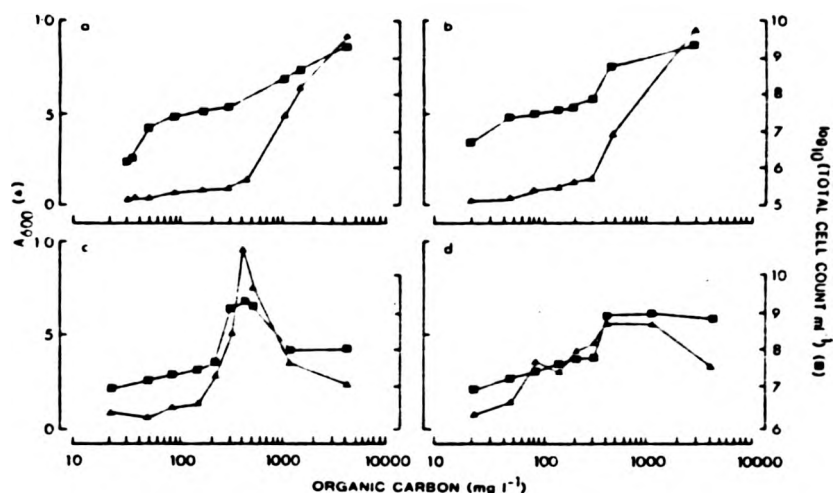


FIGURE 3.12 - Responses of "model" and freshly isolated prosthecate bacteria to varying peptone concentrations. (a) The "model copiotroph" Escherichia coli C. (b) the "model oligotroph" Caulobacter crescentus CB15. (c) the Hyphomonas-like isolate 9/S2. (d) the Hyphomonas-like isolate B/wg1. Organisms were cultivated to the late stationary phase of growth in minimal medium (Staley, 1981a) containing variable concentrations of Difco Bacto-peptone and cell yield determined by measurements of A_{200} (Δ) and total cell count obtained by means of a Coulter Counter model ZBI (\blacksquare) operating at a 1/amplification of 0.25.

lower carbon concentrations, the yield of all organisms was approximately equal suggesting that neither of the isolates was capable of a more efficient use of nutrients in the production of new cells than the two model organisms.

Whilst it appears that the isolate 9/S2 in some way resembles the obligate oligotrophs of other workers, it must be emphasised that Martin & MacLeod (1984) have shown that the inhibition of marine oligotrophic bacteria by increased concentrations of organic carbon is carbon-source dependent. In order to investigate the possibility of this occurring in this case, the four test organisms were cultivated in media containing defined carbon sources at concentrations that, in the light of the above observations, were close to the optimal for the oligotrophic-type of response or potentially inhibitory. The results obtained (Table 3.2) demonstrate that the inhibitory response of high organic carbon concentrations is also source-dependent in fresh water isolates. Whilst both E. coli and C. crescentus exhibited direct proportionality between organic carbon concentration and cell yield with all compounds tested both, of the Hyphomonas-like isolates were sensitive to some compounds but not to others. For example, isolate 9/S2 was markedly inhibited by peptone but not by glucose, galactose or citrate and isolate 8/wg1 was markedly inhibited by glucose and somewhat inhibited by peptone but was not affected by galactose at identical concentrations.

These results are in agreement with those of Martin & MacLeod (1984) which showed that the manifestation of

Table 3.2 - Responses of model bacteria and freshly isolated prosthecae bacteria to varying concentrations of defined carbon sources. Cultures were grown to the early stationary phase of growth and yield determined by measurements of A_{400} . NG = no growth.

<u>Carbon source</u>	<u>Final A_{400}</u>			
	<u>E.coli</u>	<u>CB15</u>	<u>9/S2</u>	<u>8/wg1</u>
Glucose 100mg l ⁻¹	.075	.085	.093	.102
Glucose 10000mg l ⁻¹	.923	.900	.100	.039
Galactose 100mg l ⁻¹	.051	.130	.020	.040
Galactose 10000mg l ⁻¹	.491	.370	.220	.045
Citrate 100mg l ⁻¹	.045	.043	.015	NG
Citrate 10000mg l ⁻¹	.655	.675	.045	NG
Succinate 100mg l ⁻¹	.020	.020	NG	.040
Succinate 10000mg l ⁻¹	.470	.200	NG	.018

oligotrophy in laboratory culture is dependent upon the test system employed. Whilst the optimum concentrations of carbon sources for the "oligotrophic" isolates were somewhat higher than those normally considered as oligotrophic, these values and the levels at which inhibition occurred are very similar to those reported in the literature for marine and soil isolates (Ishida & Kadota, 1981; Hattori & Hattori, 1980; Akagi *et al.*, 1980). Indeed, although in these cases the isolates were classified as oligotrophic, many were capable of growth on media containing between 1 and 5g organic carbon l⁻¹. It must be emphasised that measurements of optical density are not ideally suited to this type of investigation.

For turbidity to be measurable with a typical spectrophotometer approximately 10^6 cells ml^{-1} must be present (Akagi et al., 1980) which requires an organic carbon concentration of approximately 2mg l^{-1} (Martin & MacLeod, 1984). Whilst in this study the levels of organic carbon employed were higher than this and the use of total cell count data confirmed the absorbance data, these factors demonstrate that optical density measurements cannot be relied on in studies of oligotrophic bacteria at very low nutrient levels. Indeed, since turbidity can be produced by oligocarbophilic growth (Geller, 1983) the values reported in this study may be somewhat higher than would be directly produced from the organic carbon levels present in the media. However, the provision of carbon from such sources should be at a continuous but very low concentration and should not significantly alter the nature of the responses observed.

3.1.5) Conclusions

These results demonstrate that prosthecate bacteria are successful in the oligotrophic fresh water environment. These organisms, however, are by no means the only bacteria found in such environments and exist in a population that includes a variety of morphologically "typical" bacterial types. These studies confirm the potential importance of interfaces in oligotrophic environments but there is no evidence to confirm the proposition that nutrient sensitivity patterns show that free-living organisms are obligate oligotrophs whereas attached forms are facultative (Ishida et al., 1980; Ishida

& Kadota, 1981). The responses of the bacteria observed in these studies both in situ and in axenic culture support the suggestion that the present classification of bacteria into trophic groups on the basis of organic carbon concentration is naive (Martin & MacLeod, 1984).

Many of the observed forms and the isolates obtained possessed a number of potential adaptations for growth in the oligotrophic environment. The role of attachment has already been noted and most of the bacteria examined were motile. A significant proportion possessed intracellular nutrient storage bodies. With respect to the prosthecate bacteria it is interesting to note the existence of swarmer cell stages and the long prosthecae observed. These factors in particular have been proposed to be especially important in the adaptation of prosthecate bacteria to an oligotrophic mode of existence, the former as a means of increasing the surface area to volume ratio, and hence maximising nutrient transport potential (Hirsch, 1979; Poindexter, 1981a; 1981b), and the latter as metabolically repressed survival and dispersal stages (Dow et al., 1983). As a consequence of the difficulties in obtaining true oligotrophic bacteria for investigations of these phenomena, laboratory strains of prosthecate bacteria were employed in subsequent experiments.

3.2) THE EFFECTS OF NUTRIENT CONCENTRATION ON CELL MORPHOLOGY AND CELL TYPE EXPRESSION

As a consequence of the observations that prosthecae and

swarmer cells may play an important role in the success of prosthecate bacteria in oligotrophic environments, the alteration of nutrient conditions should bring about differing degrees of expression of these two visible phenomena in prosthecate bacteria (Poindexter, 1984a; 1984b; Schmidt & Stanier, 1966; Dow et al., 1983). In this section data are presented demonstrating variations in the expression of both potential adaptations in the prosthecate bacteria Hyphomicrobium X, Caulobacter crescentus CB15 and Rhodomicrobium vannielii Rm5 as a consequence of nutrient concentration in pure culture.

3.2.1) Population development in batch culture

The behaviour of Hyphomicrobium X in batch culture was examined under aerobic conditions at 30°C in standard HM medium (100mM methylamine; 10mM total phosphate). The growth curve (Fig. 3.13) was followed by measurements of A_{400} and total cell count. The doubling time (t_d) observed for the culture was approximately 10.5 hours, a value close to that previously reported for this organism (Attwood & Harder, 1977). The relative proportions of swarmer and prosthecate reproducing cells varied throughout the growth cycle (Fig. 3.13); prior to the onset of active growth the proportion of swarmer cells in the population was high and that of reproducing cells was low; during exponential growth the proportion of swarmer cells was greatly decreased and there was a marked increase in the occurrence of reproducing cells; during the late exponential and early stationary

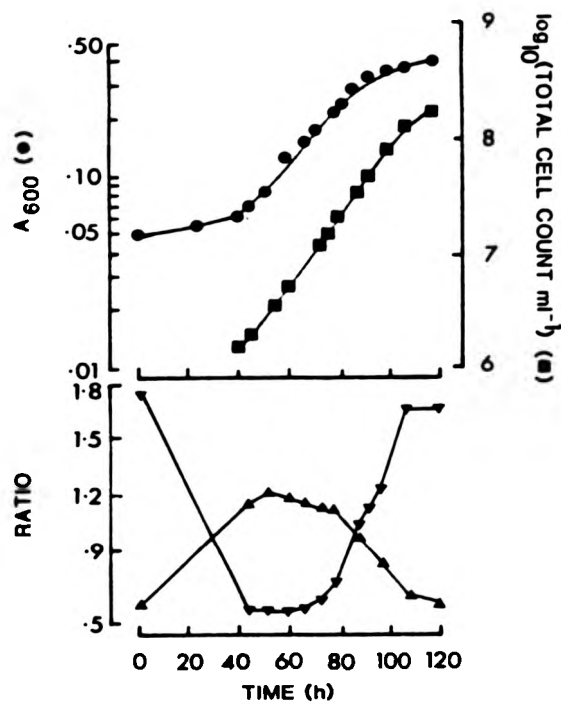


FIGURE 3.13 - Cell type expression during batch culture of *Hyphomicrobium* X. The upper graph illustrates the growth curve in 100ml portions of HM medium containing 100mM methylamine under aerobic conditions at 30°C as measured by A_{600} (●) and total cell counts (■) determined using a Coulter Counter model ZBI operating at a 1/amplification of 0.25. The lower graph illustrates the ratios of reproducing to non-reproducing prosthecate cells (▲) and swarmer to non-reproducing prosthecate cells (▼) as determined by direct cell type counts.

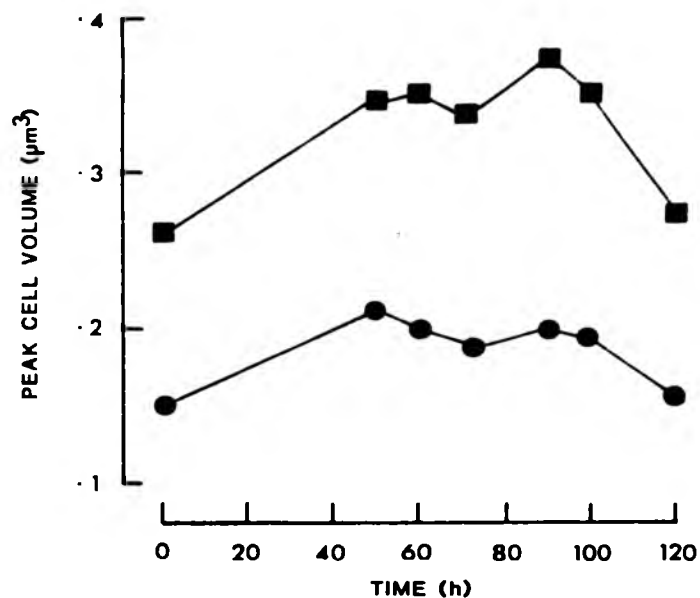


FIGURE 3.14 - Variation in peak cell volume of *Hyphomicrobium X* during batch culture. The peak cell volume of swarmer cells (●) and prosthecate cells (■) was determined using a Coulter Counter ZBI/C1000 Channelyzer system linked to an Acorn BBC model B microcomputer.

phases of growth the relative proportions of these two cell types rapidly reverted to the proportions observed during the lag phase. There was also a variation observed in the peak cell volumes of both the swarmer and prosthecate cells during the growth curve (Fig. 3.14). Both the swarmer and prosthecate cell peaks increased in volume during exponential growth and reverted to values approximating those in the inocula with the onset of the stationary phase.

Caulobacter crescentus CB15 exhibited similar behaviour when grown aerobically at 30°C in HMG medium containing 0.02% (w/v) glucose (1.1mM organic carbon source; 10mM inorganic phosphate). The observed t_d was approximately 5 hours. A variation in the peak cell volumes of the two cell types was observed during the growth curve (Fig. 3.16) but the change observed in the the swarmer cell peak fraction was minimal.

Under the conditions routinely employed for cultivation in this study, Rhodomicrobium vannielii Rm5 tended to produce chains of prosthecate cells and it is therefore difficult to classify cells into the distinct groupings employed above. Porter (1984) demonstrated that the proportion of swarmer cells was at its lowest during exponential growth and was maximal during the lag and stationary phases, a situation identical to that observed for Hyphomicrobium X and C. crescentus. Under the conditions routinely employed in this study, PM medium containing 5mM inorganic phosphate incubated at 30°C with a light intensity of approximately 1000lux, the observed t_d was

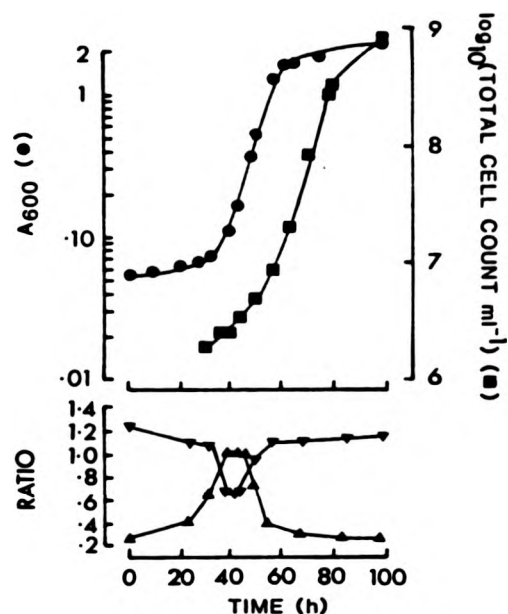


FIGURE 3.15 - Cell type expression during batch culture of *Caulobacter crescentus* CB15. The upper graph illustrates the growth curve in 100ml portions of HMB medium containing 0.02% (w/v; 1.1mM) glucose under aerobic conditions at 30°C as measured by A_{600} (●) and total cell counts (■) determined using a Coulter Counter model ZBI operating at a 1/amplification of 0.25. The lower graph illustrates the ratios of reproducing to non-reproducing prosthecae cells (▲) and swarmer to non-reproducing prosthecae cells (▼) as determined by direct cell type counts.

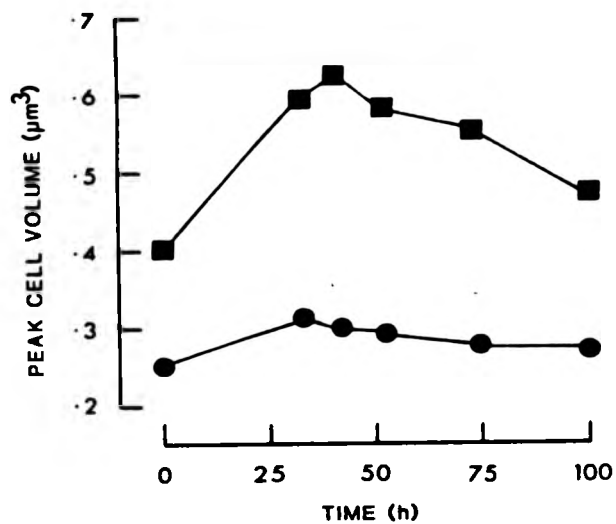


FIGURE 3.16 - Variation in peak cell volume of *Caulobacter crescentus* CB15 during batch culture. The peak cell volume of swarmer cells (●) and prosthecate cells (■) was determined using a Coulter Counter ZBI/C1000 Channelyzer system linked to an Acorn BBC model B microcomputer.

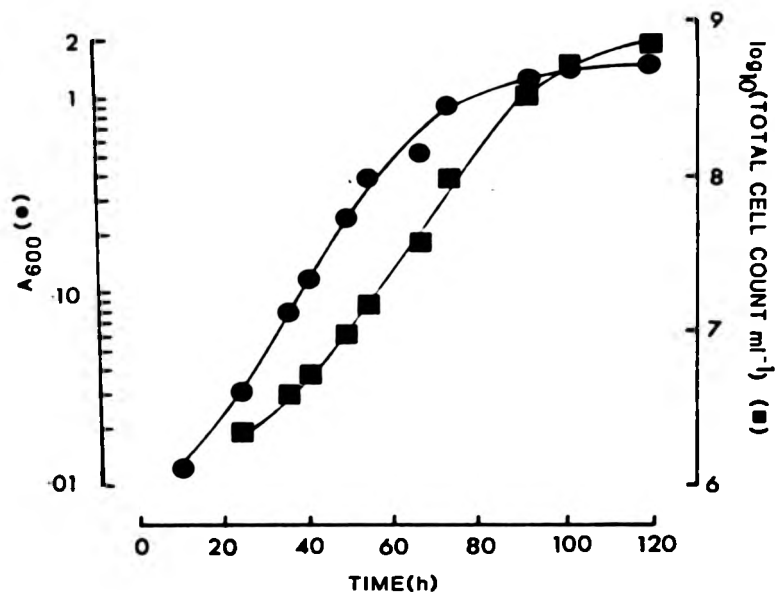


FIGURE 3.17 - Growth curve of *Rhodospirillum rubrum*
 Rm5. The organism was grown in 100ml portions of PM medium at
 30°C with a light intensity of approximately 1000lux.
 Growth was determined by measurements of A_{600} (●) and
 total cell counts (■) determined using a Coulter Counter
 model ZBI operating at a 1/amplification of 0.5. At this
 amplification long chains of cells are not detected and the
 values presented may underestimate the true cell numbers.

approximately 11 hours (Fig. 3.17), a value similar to that previously reported (Whittenbury & Dow, 1977).

The accumulation of swarmer cells during the late exponential and early stationary phases of growth has been taken to be indicative of their role as survival cell types (Dow et al., 1983). Indeed, their numbers were lowest during active growth and reproduction when the numbers of reproducing cells were highest which suggests that swarmer cells produced by cell division at these times rapidly underwent differentiation to produce reproductive cells. However, as nutrient stress ensued, the swarmer cells were maintained as undifferentiated entities. Under such conditions swarmer cells can be prevented from differentiating if an energy source and/or nutrients are not available (Dow et al., 1983; Porter, 1984).

The observations concerning peak cell volume data obtained using the Coulter Counter are somewhat in contradiction to those reported for Hyphomicrobium X by Lawrence (1978). She reported that peak cell volume of swarmer cells markedly increased during the exponential phase of growth whereas that of prosthecae cells was significantly decreased. Whilst a small increase in swarmer cell peak volume was observed in this study during the exponential growth phase, the values obtained for the prosthecae cell fraction significantly increased. Whilst the former may represent a slight increase in daughter cell size as a consequence of active growth, it would be expected that the volume of the prosthecae cell population would increase as a

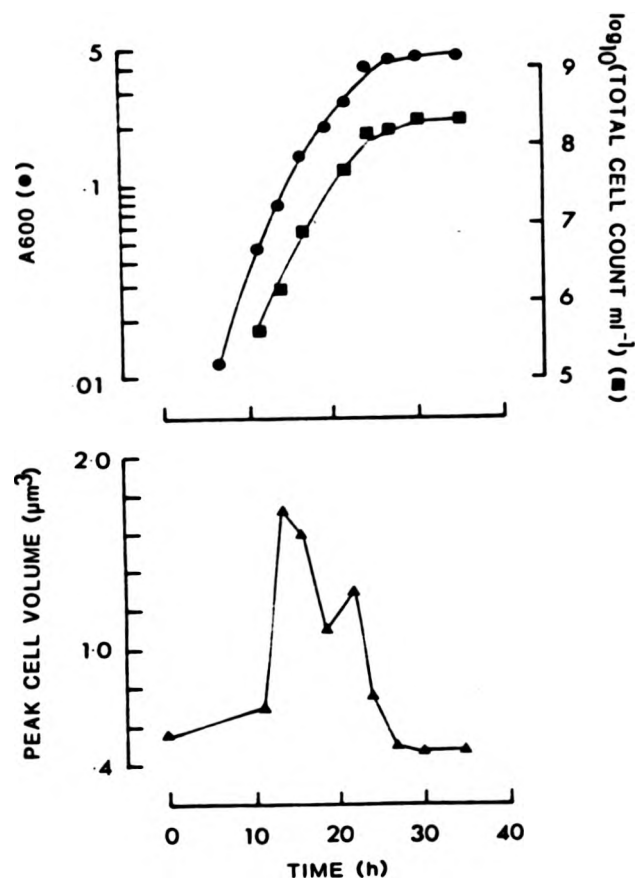


FIGURE 3.1B - Variation in peak cell volume of *E. coli* C during batch culture. The organism was grown aerobically at 30°C in 100ml portions of M9 medium containing 0.02% (w/v; 1.1mM) glucose. The upper graph illustrates the growth curve as measured by A_{600} (●) and total cell counts (■) obtained using a Coulter Counter model ZBI operating at a 1/amplification of 0.25. The lower graph illustrates peak cell volume determined using a Coulter Counter model ZBI/C1000 Channelyzer system linked to an Acorn BBC model B microcomputer.

consequence of cell growth and daughter cell formation. Such a response was observed in E. coli C growing aerobically at 30°C in M9 medium containing 0.02% (w/v) glucose (t_l approximately 2 hours; Fig. 3.18) and has previously been reported in E. coli K12 growing in a medium containing 0.2% (w/v) glucose (Kahru & Vilu, 1983) where it was suggested that this reflected synthesis of new cell material during active growth. It is therefore probable that the shift in peak cell volume observed in these experiments is a result of bulk cell growth during the exponential phase since alterations in prostheca length were not observed during this time. However, it must be emphasised that the changes observed in Hyphomicrobium X and C. crescentus were comparatively small when compared to those observed for E. coli and the former may be accentuated by inaccuracies inherent in the Coulter Counter over such a narrow range (Trueba et al., 1982).

3.2.2) Morphological responses to varying carbon and phosphate concentrations in batch culture

Hyphomicrobium X was cultivated as described above in media containing various concentrations of methylamine and phosphate. Figure 3.19 illustrates the yields (expressed in terms of total cell count) of this organism in media containing 10mM phosphate and variable concentrations of methylamine. Maximum cell numbers were obtained in medium containing 100mM methylamine and at higher concentrations growth was somewhat inhibited. Between approximately 10 and

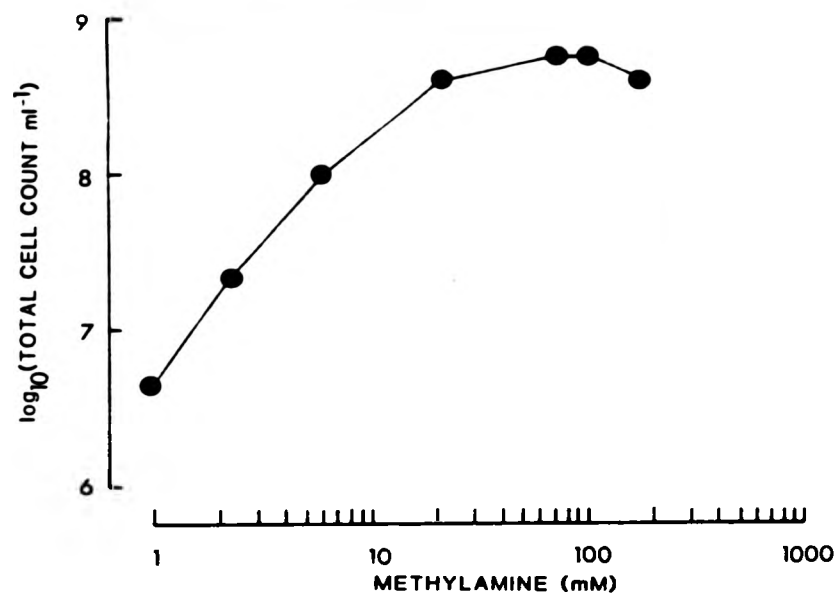


FIGURE 3.19 - Yield of Hyphomicrobium X at varying methylamine concentrations in batch culture. Cells were grown to the early stationary phase aerobically at 30°C in 100ml portions of HM medium containing various concentrations of methylamine. Total cell count was determined using a Coulter Counter model ZBI operating at a 1/amplification of 0.25.

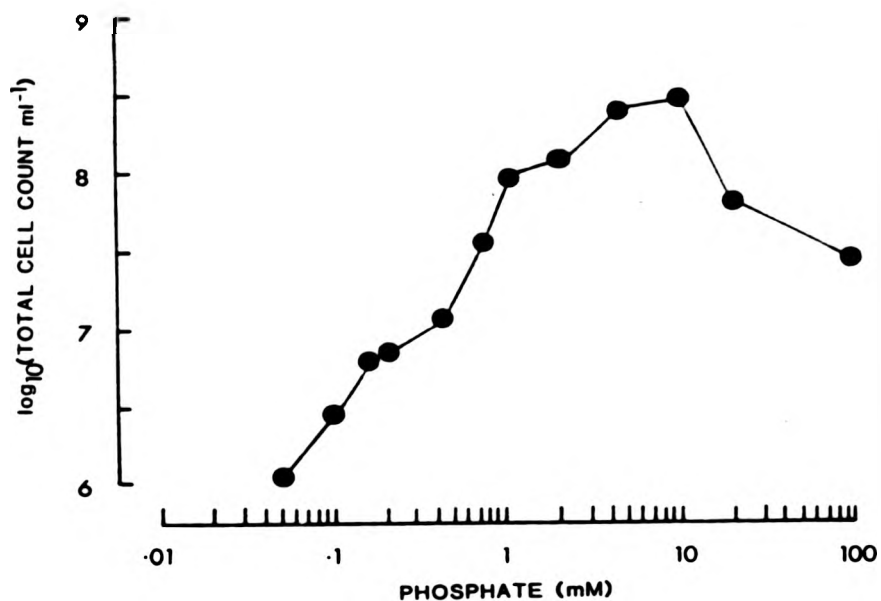


FIGURE 3.20 - Yield of Hyphomicrobium X at varying inorganic phosphate concentrations in batch culture. Cells were grown to the early stationary phase aerobically at 30°C in 100ml portions of HM medium containing various concentrations of phosphate. Total cell count was determined using a Coulter Counter model ZBI operating at a 1/amplification of 0.25.

100mM methylamine, the yield remained relatively constant suggesting that carbon was not the sole limiting nutrient. The optimum concentration of phosphate in media containing 100mM methylamine (Fig. 3.20) was 10mM with growth being inhibited at concentrations above this. Between 1 and 10mM phosphate the yield remained relatively constant. Decreasing concentrations of both nutrients resulted in increased prostheca length. Variations in carbon concentrations (Fig. 3.21) yielded an approximately linear response over the range tested. The response to decreasing phosphate concentrations (Fig. 3.22) was most marked below 1mM, the level at which phosphate was apparently limiting. No significant difference between carbon and phosphate effects on prostheca length was evident over the concentrations tested.

C. crescentus exhibited a similar response. The maximum yield in media containing 10mM phosphate was at approximately 100mM glucose with a marked decrease in yield below 1mM (Fig. 3.23). Yield was very significantly inhibited at 200mM glucose. In media containing 1.1mM glucose, maximum yield was obtained at 10mM phosphate with some inhibition observed at higher concentrations (Fig. 3.24). Yield was significantly reduced below approximately 1mM phosphate. Prostheca length increased with decreasing levels of both carbon (Fig. 3.25) and phosphate (Fig. 3.26) although in the latter case significant effects were only apparent below 1mM.

Of the three organisms tested, it was R. vannielii that exhibited the clearest responses to varying phosphate concentrations. Yield was significantly dependent on

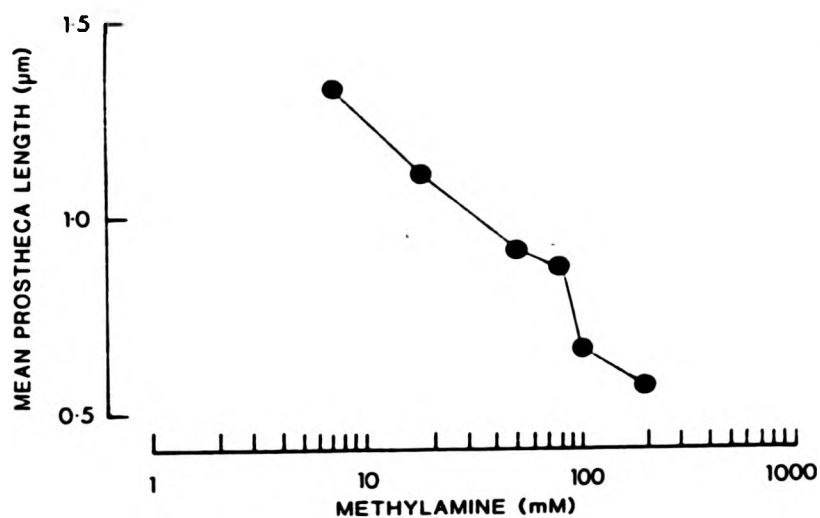


FIGURE 3.21 - Prostheca length in Hyphomicrobium X at varying methylamine concentrations in batch culture. Cells were grown to the early stationary phase aerobically at 30°C in 100ml portions of HM medium containing various concentrations of methylamine. Prostheca length was determined by measurements of in excess of 100 cells in electron microscope preparations.

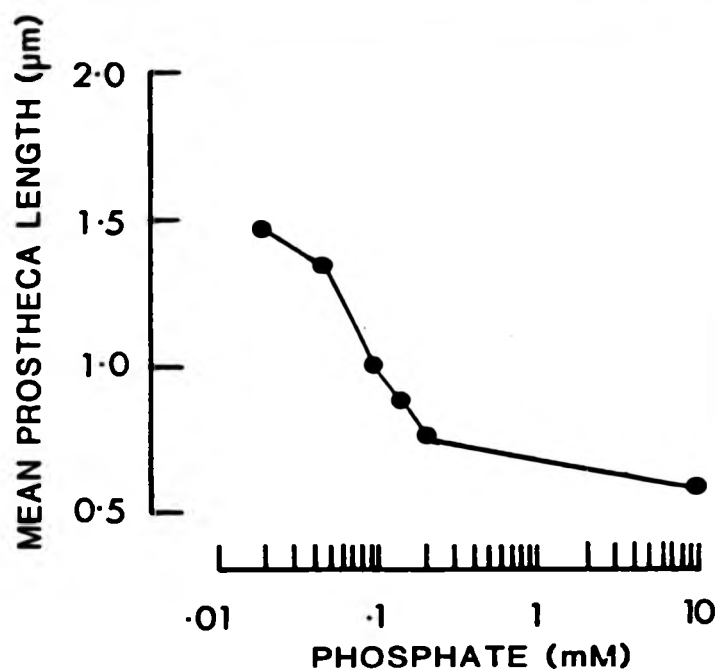


FIGURE 3.22 - Prostheca length in *Hyphomicrobium* X at varying inorganic phosphate concentrations in batch culture. Cells were grown to the early stationary phase aerobically at 30°C in 100ml portions of HM medium containing various concentrations of phosphate. Prostheca length was determined by measurements of in excess of 100 cells in electron microscope preparations.



FIGURE 3.23 - Yield of *Caulobacter crescentus* CB15 at varying glucose concentrations in batch culture. Cells were grown to the early stationary phase aerobically at 30°C in 100ml portions of HMB medium containing various concentrations of glucose and total cell count determined using a Coulter Counter model ZBI operating at a 1/amplification of 0.25.

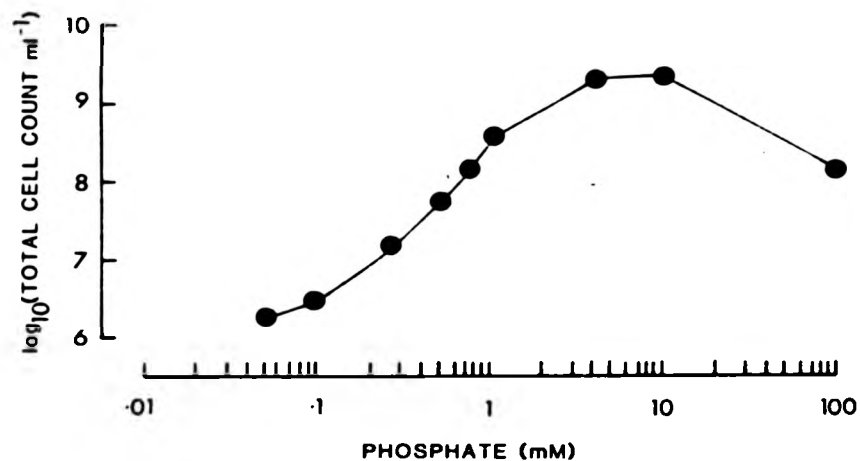


FIGURE 3.24 - Yield of Caulobacter crescentus CB15 at varying inorganic phosphate concentrations in batch culture. Cells were grown to the early stationary phase aerobically at 30°C in 100ml portions of HMB medium containing various concentrations of phosphate. Total cell count was determined using a Coulter Counter model ZBI operating at a 1/amplification of 0.25.

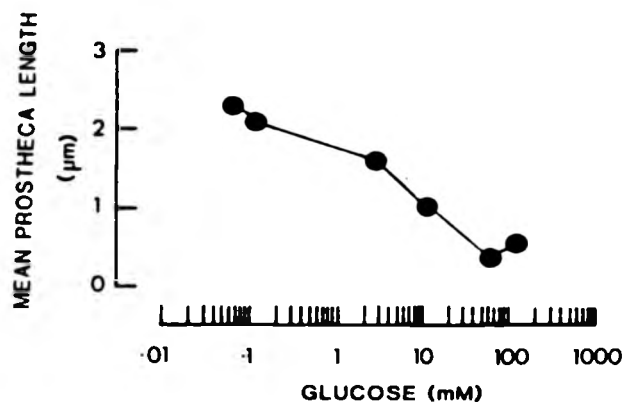


FIGURE 3.25 - Prostheca length in *Caulobacter crescentus* CB15 at varying glucose concentrations in batch culture. Cells were grown to the early stationary phase aerobically at 30°C in 100ml portions of HMG medium containing various concentrations of glucose. Prostheca length was determined by measurements of in excess of 100 cells in electron microscope preparations.

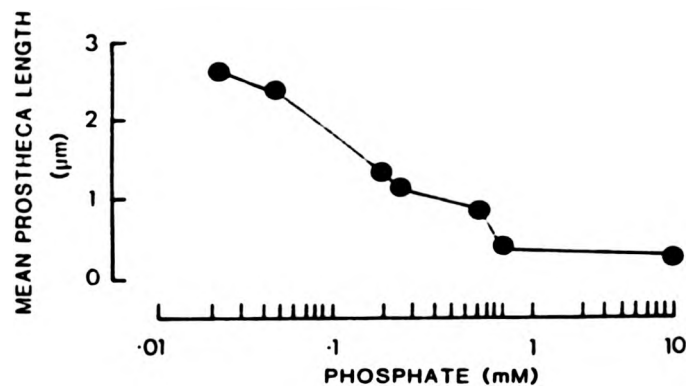


FIGURE 3.26 - Prostheca length in *Caulobacter crescentus* CB15 at varying inorganic phosphate concentrations in batch culture. Cells were grown to the early stationary phase aerobically at 30°C in 100ml portions of HMB medium containing various concentrations of phosphate. Prostheca length was determined by measurements of in excess of 100 cells in electron microscope preparations.

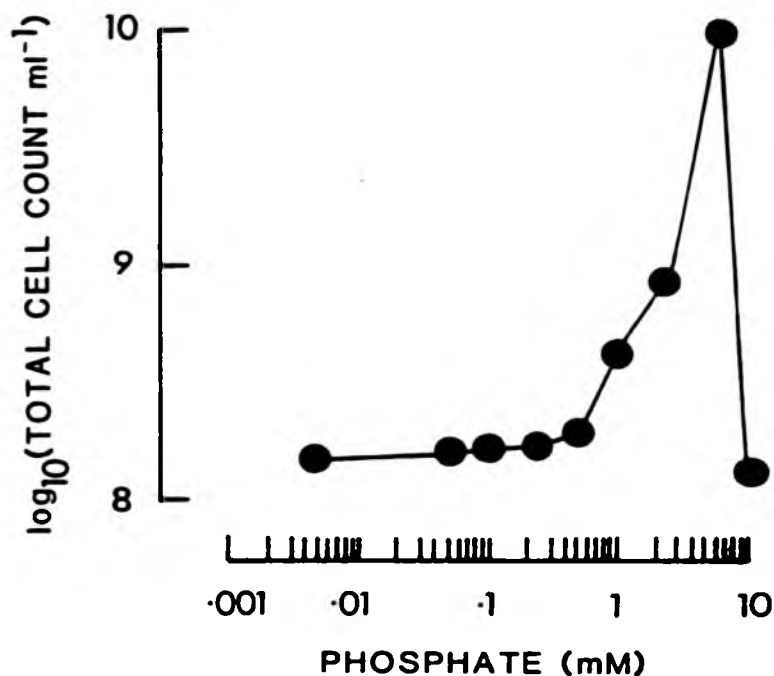


FIGURE 3.27 - Yield of *Rhodospirillum rubrum* Rm5 at varying inorganic phosphate concentrations in batch culture. Cells were grown to the early stationary phase at 30°C with a light intensity of approximately 1000lux in 100ml portions of PM medium containing various concentrations of phosphate. Total cell count was determined using a Coulter Counter model ZBI operating at a 1/amplification of 0.5.

concentration (Fig. 3.27) and the highest cell count was obtained at 5mM. Very significant inhibition of growth was observed at higher phosphate concentrations and no growth occurred at 20mM. The concentration of phosphate also had very marked effects on prostheca length at all concentrations tested (Fig. 3.28) with very long prosthecae produced at lower concentrations. This clear pattern of behaviour is illustrated by electron micrographs in Figure 3.29. At 0.01mM phosphate, chains of cells are produced which possess very long prosthecae, whereas with increasing phosphate concentration the intercellular distances are decreased. The inhibitory effect of 10mM phosphate is clearly illustrated in Figure 3.29c with the cells possessing distorted cell bodies and short prosthecae.

Nutrient effects on the yields of prosthecate bacteria normally employed in laboratory investigations have previously received detailed investigation only with respect to phosphate effects on Caulobacter. Grula et al. (1954) reported that newly isolated C. vibrioides grew optimally at phosphate concentrations of between 0.9 and 14.3mM, results which were confirmed by Schmidt & Stanier (1966) working with C. crescentus and endorsed by this study. Poindexter (1979) reported that yields of C. crescentus CB2 at 1mM phosphate were 65-67% of those obtained at 0.1mM and further stated that Caulobacter was characterised by having low upper limits of organic carbon and phosphate tolerance (Poindexter, 1981b). Whilst these observations are somewhat in contradiction to both my own and

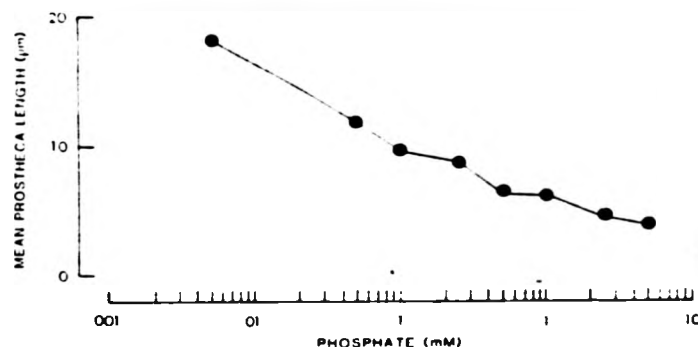


FIGURE 3.28 - Prostheca length in Rhodospirillum
vannielii Rm5 at varying inorganic phosphate
concentrations in batch culture. Cells were grown to the
early stationary phase at 30°C with a light intensity of
approximately 1000lux in 100ml portions of PM medium
containing various concentrations of phosphate. Prostheca
length was determined by measurements of in excess of 100
cells in electron microscope preparations.



FIGURE 3.29 - Effects of inorganic phosphate on *Rhodospirillum rubrum* Rm5. Cells were grown to the early stationary phase at 30°C with a light intensity of approximately 1000lux in 100ml portions of PM medium containing various concentrations of phosphate. (a) Cells grown in 0.1mM phosphate. (b) Cells grown in 5mM phosphate. (c) Cells grown in 10mM phosphate. Electron micrographs of phosphotungstic acid stained specimens. Scale bar = 1.0μm.

earlier data, it is undoubtedly true that the most important consideration in all experiments designed to study nutrient effects on "oligotrophic" bacteria is not the absolute levels of individual nutrients but the balance of nutrients in the media and that experiments to study such effects would be most complex (Poindexter, 1981b). Basic studies on growth yields, although pertinent to published concepts of oligotrophy, do not provide information regarding the growth rates of the organisms under various nutrient conditions. Such data are important in understanding the competitiveness of prosthecae bacteria under oligotrophic conditions but this was not investigated in detail here. Poindexter (1981b; 1984a) demonstrated that the growth rate of Caulobacter crescentus CB2 was not greatly altered over a wide range of organic carbon and phosphate concentrations and observations made during this study apparently confirmed these quantitative data.

The yields of all three species examined were similar and clear optimum and maximum concentrations of both carbon and phosphate observed. The clearest reaction to increased nutrient concentrations was that of R. vannielii to phosphate which was markedly inhibited at the highest concentrations tested. However, the concentrations of both phosphate and organic carbon tested for all organisms were generally higher than those considered to be oligotrophic. All optima were greatly above the scale of 1-15 μ g organic carbon l^{-1} (0.0001-0.0015% (w/v)) proposed by Kuznetsov et al. (1979) as being characteristic of obligate

oligotrophs, although all three were capable of growth at such levels of organic carbon and the presumed analogous phosphate concentrations. Furthermore, the maxima obtained were all very much above the levels proposed for oligotrophs. These data therefore indicate that extreme caution is necessary when comparing observations made using laboratory strains of prosthecate bacteria, in the media usually employed, to bacterial behaviour in the oligotrophic natural environment.

The elongation of prosthecae with decreasing nutrient concentration has been clearly documented and has been proposed as a mechanism for increasing the surface area to volume ratio of the prosthecate bacteria and hence maximising nutrient uptake ability. Schmidt & Stanier (1966) reported that cultivation of C. crescentus in dilute media resulted in the production of prosthecae several times longer than those produced in undiluted media and that it was the concentration of phosphate that had the most significant effect. This was confirmed by Haars & Schmidt (1974) who used phosphate-limited continuous cultures of Caulobacter. The same response of Rhodomicrobium (Whittenbury & Dow, 1977) and Hyphomicrobium (Harder & Attwood, 1978; Poindexter, 1984a) to decreased nutrient concentrations had also been noted. However, Poindexter (1979; 1984a; 1984b) has used these observations as the basis for a model that proposes that prosthecae function as specialised phosphate uptake sites. The formulation of this concept relied upon the maximal stimulation of prostheca growth by decreased

phosphate concentrations and depleted ATP levels (Poindexter, 1979). The data presented herein and a significant proportion of those previously reported contradict this model. Whilst phosphate does indeed stimulate prostheca development, decreasing organic carbon concentrations in the presence of excess phosphate do so also. The experiments reported here demonstrate only a slight difference in the magnitude of the effect caused by organic carbon and phosphate over the range of concentrations tested. As I shall discuss below there also exist a number of other lines of evidence against this hypothesis, yet even on the basis of the ecological and morphological investigations discussed above, although it is probable that elongated prosthecae function, at least in part, for increasing available surface area, it appears most unlikely that they function solely for phosphate uptake.

3.2.3) Cell type expression as a consequence of carbon and phosphate concentration in batch culture

The populations of Hyphomicrobium X and C. crescentus CB15 present at the early stationary phase of growth in media containing various concentrations of organic carbon and phosphate were examined in order to ascertain the relative proportions of swarmer cells present. With increasing organic carbon concentrations Hyphomicrobium X (Fig. 3.30) exhibited a decreasing proportion of swarmer cells, which was particularly marked at the lower concentrations tested, and some increase in the proportion of reproducing cells. The effects of increasing phosphate concentration on this

organism (Fig. 3.31) were similar in nature although less marked. C. crescentus exhibits little change in the relative proportions of swarmer cells and reproducing cells with increasing carbon concentrations (Fig. 3.32). However, increasing phosphate concentrations (Fig. 3.33) resulted in a significant increase in the proportion of swarmer cells present in the population.

These results extend the concept discussed above (Section 3.2.1) that the accumulation of swarmer cells during nutrient stress is indicative of their possible roles as survival and dispersal cells. Poindexter (1981b; 1984a) reported that for C. crescentus CB2 and Hyphomicrobium T37 the proportion of swarmer cells in the population in the stationary phase was directly proportional to the phosphate concentration in the medium: low phosphate concentrations resulted in very low numbers of swarmer cells present whereas high concentrations resulted in the presence of very high numbers of swarmer cells. These results were questioned by Dow et al. (1983). Poindexter (1984a) proposed that the inhibition of swarmer cell differentiation was a consequence of the levels of inorganic phosphorus "inherited" from the mother cells. When cultivated in low phosphate medium the swarmer cells "inherit" little phosphate from their mother cells and, since the prosthecae are proposed to function as specialised phosphate uptake cells, the swarmer cells rapidly undergo differentiation with consequent prostheca synthesis in order to ensure a continuing phosphate supply for metabolic processes. In contrast, swarmer cells from

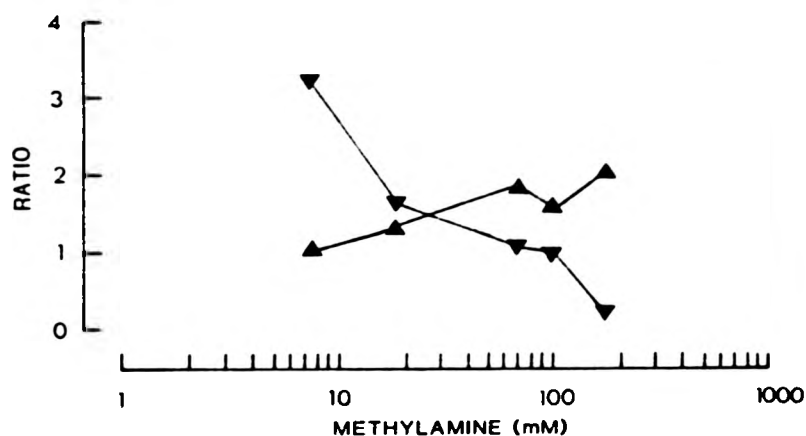


FIGURE 3.30 - Cell type expression of Hyphomicrobium X at varying methylamine concentrations in batch culture. Cells were grown to the early stationary phase aerobically at 30°C in 100ml portions of HM medium containing various concentrations of methylamine. Cell numbers were ascertained by counts of in excess of 300 cells in electron microscope preparations. (▼) Ratio of swarmer cells to non-reproducing prosthecae cells. (▲) Ratio of reproducing to non-reproducing prosthecae cells.

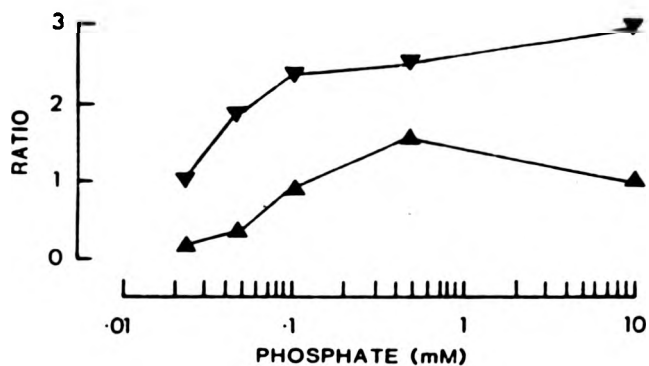


FIGURE 3.31 - Cell type expression of Hyphomicrobium X at varying inorganic phosphate concentrations in batch culture. Cells were grown to the early stationary phase aerobically at 30°C in 100ml portions of HM medium containing various concentrations of phosphate. Cell numbers were ascertained by counts of in excess of 300 cells in electron microscope preparations. (▼) Ratio of swarmer cells to non-reproducing prosthecae cells. (▲) Ratio of reproducing to non-reproducing prosthecae cells.

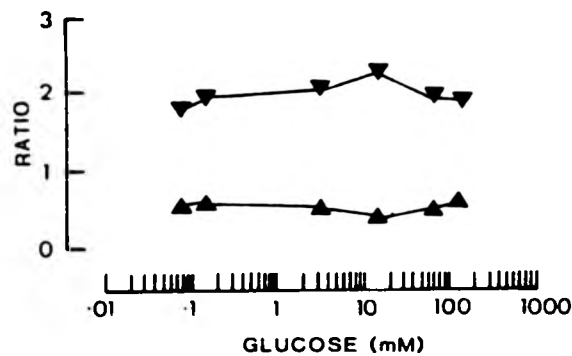


FIGURE 3.32 - Cell type expression of *Caulobacter crescentus* CB15 at varying glucose concentrations in batch culture. Cells were grown to the early stationary phase aerobically at 30°C in 100ml portions of HMG medium containing various concentrations of glucose. Cell numbers were ascertained by counts of in excess of 300 cells in electron microscope preparations. (▼) Ratio of swarmer cells to non-reproducing prosthecae cells. (▲) Ratio of reproducing to non-reproducing prosthecae cells.

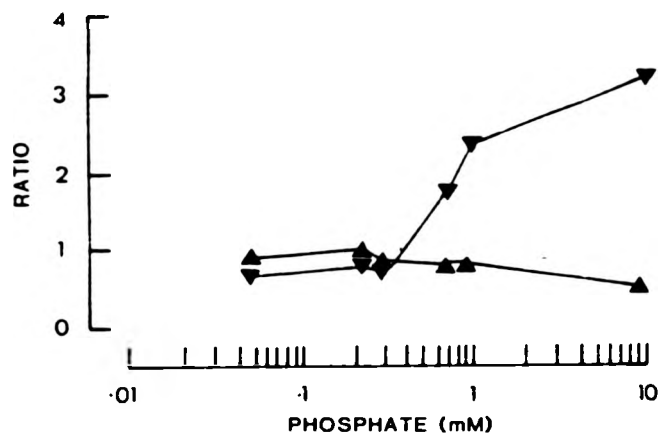


FIGURE 3.33 - Cell type expression of Caulobacter crescentus CB15 at varying inorganic phosphate concentrations in batch culture. Cells were grown to early stationary phase aerobically at 30°C in 100ml portions of HMB medium containing various concentrations of phosphate. Cell numbers were ascertained by counts of in excess of 300 cells in electron microscope preparations. (▼) Ratio of swarmer cells to non-reproducing prosthecae cells. (▲) Ratio of reproducing to non-reproducing prosthecae cells.

phosphate-rich media "inherit" high levels of phosphate and are therefore viable in the undifferentiated state for longer periods of time. Whilst in this study there is some evidence to confirm the observations of Poindexter (1981b; 1984a) with respect to increasing relative numbers of swarmer cells with increasing phosphate concentrations in C. crescentus, the effects were less marked than those reported and were not significant in Hyphomicrobium X. Furthermore, observations over relatively long periods on swarmer cells of Hyphomicrobium, Caulobacter and Rhodomicrobium (Lawrence, 1978; Dow et al., 1983; Porter, 1984) gave no indication that the cells undergo differentiation unless an energy source is supplied.

3.2.4) Effects of calcium concentration on prostheca development

The model of Poindexter (1984a; 1984b), assigning to prosthecae the function of specialised phosphate uptake sites, was based to a large extent on observations of the effects of calcium on prostheca length in batch culture. Increased prostheca length, in addition to being a direct function of phosphate concentration, resulted from increased calcium levels in media due to variations in the absolute amounts added or to the addition of EGTA, a specific calcium chelator. In order to investigate these effects, Hyphomicrobium X, C. crescentus and R. vannielii were cultivated in media containing various levels of calcium.

All organisms (Table 3.3) responded only very slightly

TABLE 3.3 - Effects of calcium on prostheca length.

Organisms were grown to the early stationary phase in media containing varying concentrations of phosphate and calcium. Growth yield was determined using a Coulter Counter model ZBI. Prostheca length was determined from measurements of in excess of 100 cells in electron microscope preparations. ND = not determined.

Calcium (mM)	Phosphate (mM)	Total cell count (ml ⁻¹)	Mean prostheca length (μm)
<u>Hyphomicrobium X</u>			
0.020	0.15	1.18x10 ⁷	0.86
0.041	0.15	1.34x10 ⁷	0.88
0.200	0.15	1.21x10 ⁷	0.86
0.400	0.15	1.09x10 ⁷	1.46
<u>Caulobacter crescentus CB15</u>			
0.033	0.68	3.71x10 ⁶	1.09
0.074	0.68	2.86x10 ⁶	ND
0.238	0.68	5.11x10 ⁶	1.16
<u>Rhodomicrobium vannielii Rm5</u>			
0.160	5.00	7.99x10 ⁶	3.61
0.201	5.00	8.11x10 ⁶	3.93
0.365	5.00	5.89x10 ⁶	ND ^a

(a) Growth poor and cells distorted.

to alterations in calcium concentrations as determined by growth yield and prostheca length except for Hyphomicrobium X when cultivated at the highest calcium levels.

R. vannielii grew very poorly in media containing 0.365mM calcium and the cells produced were morphologically abnormal, suggesting that calcium has toxic effects at comparatively low concentrations on this organism. It is therefore possible

that the other bacteria tested may suffer calcium-mediated toxic effects of a less severe nature at similarly low concentrations. Further investigations using Hyphomicrobium X at a variety of calcium concentrations in media containing three different concentrations of phosphate (Fig. 3.34) demonstrated that calcium had little effect on growth yield over the range tested.

Poindexter (1984a) grew Hyphomicrobium T37 in media containing 0.05mM phosphate plus either 0 or 0.5mM calcium and obtained a significant alteration in growth rate and a doubling of prostheca length. Using C. crescentus CB2 under the same conditions a threefold increase in prostheca length was observed. These effects were proposed to be due to chemical sequestration of phosphate anions by calcium and were reduced when EGTA was used to effectively reduce the media calcium levels. The data reported above show only a minimal effect of calcium on prostheca length although the media calcium to phosphate ratios employed are less than those used by Poindexter (1984a; 1984b). It is therefore probable that the observations of Poindexter (1984a; 1984b) may not reflect responses to phosphate concentration per se but may be direct effects of calcium itself, sequestration of other ionic components or toxic effects of calcium or EGTA.

3.2.5) Environmental control of intracellular polyphosphate storage

The storage of polymeric nutrient reserve compounds is proposed to be of particular importance in ensuring survival

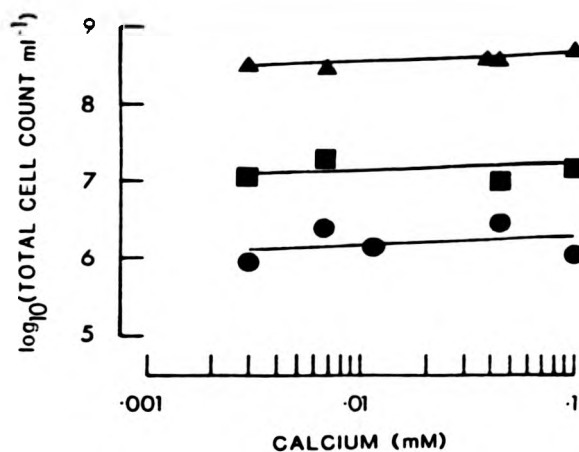


FIGURE 3.34 - Yield of Hyphomicrobium X at varying calcium concentrations in media containing three levels of phosphate. Cells were grown to the early stationary phase aerobically at 30°C in 100ml portions of HM medium containing 0.1mM (●), 0.5mM (■) or 1mM (▲) phosphate and various concentrations of calcium and total cell count determined using a Coulter Counter model ZBI operating at a 1/amplification of 0.25.

and competitiveness in the oligotrophic environment (Hirsch, 1979; Shilo, 1980; Poindexter, 1981a). Caulobacter crescentus is able to accumulate phosphate and organic carbon reserve materials, the former as polyphosphate (PP_i) and the latter as PHB (poly- β -hydroxybutyrate) and polyglucose (Poindexter, 1964). The production of these compounds in culture has been shown to be due to the presence of an excess of carbon over phosphate for PHB production and of phosphate over organic carbon for PP_i production (Poindexter & Eley, 1983). Whilst PHB production, in particular, has been studied in C. crescentus the production of PP_i in prosthecae bacteria has received less attention.

Hyphomicrobium X, C. crescentus and R. vannielii all accumulated PP_i in response to increasing phosphate concentrations in batch culture (Fig. 3.35) which is confirmatory of previous reports for Caulobacter (Poindexter & Eley, 1983; Poindexter, 1984b) and demonstrates that PP_i storage is important in a number of prosthecae bacteria. The levels of stored PP_i obtained for C. crescentus are in close agreement with those obtained by Poindexter (1984b) who obtained values of 0.3-0.6 $\mu\text{g (mg dry weight)}^{-1}$ for cells cultivated in media containing 0.25mM phosphate and 2.0-2.5 $\mu\text{g (mg dry weight)}^{-1}$ for cells cultivated in media containing 0.5mM phosphate.

3.2.6) Continuous culture of *Escherichia coli* C

This organism was chosen for investigation of the effects of

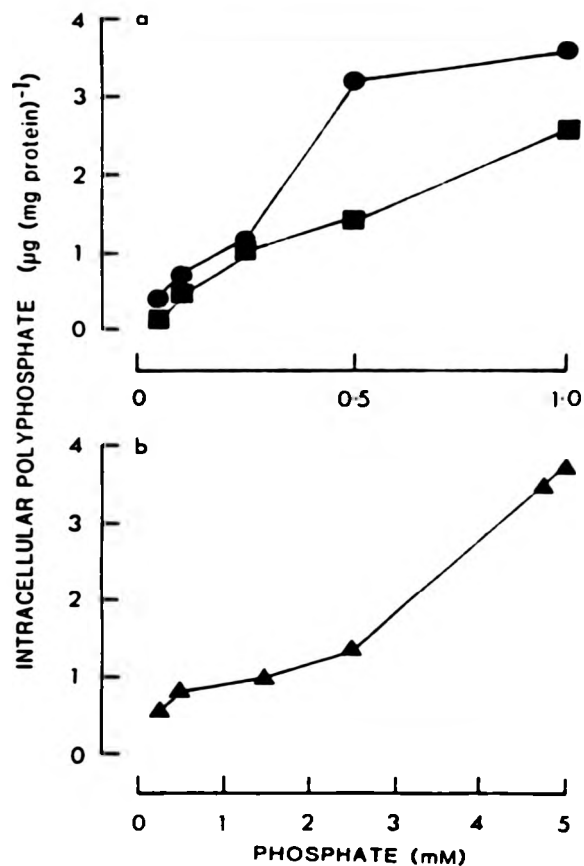


FIGURE 3.35 - Polyphosphate storage as a consequence of medium phosphate concentration. (a) *Hyphomicrobium X* (●) and *C. crescentus* CB15 (■). (b) *R. vannielii* Rm5. Cells were grown to early stationary phase in media containing various concentrations of phosphate, inorganic polyphosphate purified and hydrolysed according to the method of Poindexter & Eley (1983) and inorganic phosphate analysed by means of a Sigma Diagnostics inorganic phosphate assay kit (procedure no. 360-LV).

growth rate on cell morphology because it is a "copiotroph" and since it would provide information concerning the responses of morphologically "typical" bacteria under conditions similar to those employed for the continuous culture of Hyphomicrobium X described below. E. coli was cultivated aerobically at 30°C in M9 medium (Lark et al., 1963) containing 0.02% or 0.002% (w/v) glucose as the sole carbon and energy source. Under both conditions the culture was carbon-limited and limitation was demonstrated by increasing the glucose concentration in the medium reservoir by a factor of two and observing a consequent increase in the cell concentration in the culture vessel. Maximum growth rates (μ_{max}) were determined in both media by means of duplicate washout rate experiments (Jannasch, 1969) and were found to be $0.319h^{-1}$ (0.02% (w/v) glucose; maximum t_d 2.17h) and $0.160h^{-1}$ (0.002% (w/v) glucose; maximum t_d 4.41h). With both media there was a direct relationship between cell volume and dilution rate (Fig. 3.36): as dilution rate, and hence growth rate, increased there was a significant increase in peak cell volume which was proportional to the dilution rate itself rather than absolute organic carbon concentration.

One consequence of a reduction in cell volume is an increase in surface area to volume ratio and presumably a consequent increase in nutrient transport efficiency. The reduction of cell volume with decreasing dilution rate in continuous culture has been described for a variety of morphologically "typical" bacteria including Bacillus megaterium (Herbert, 1961) and Salmonella typhimurium

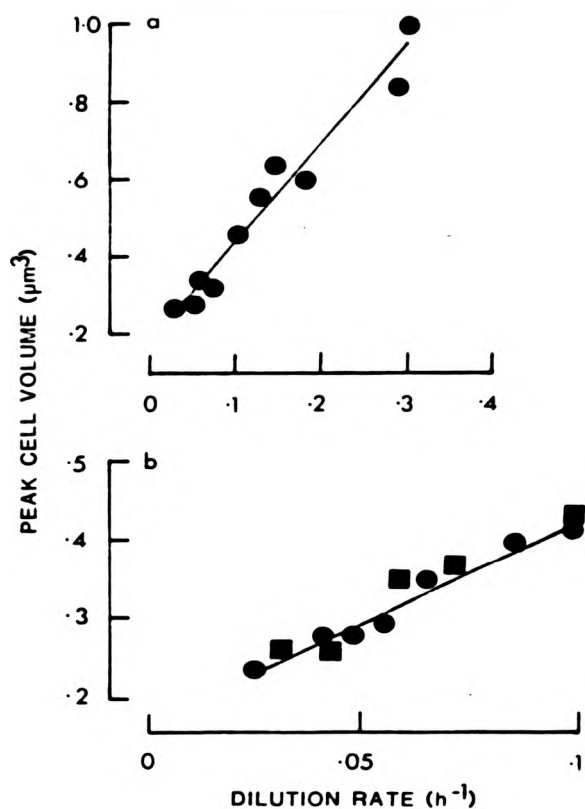


FIGURE 3.36 - Cell volume of *E. coli* C in carbon-limited continuous culture. Cells were grown to steady state at 30°C in M9 medium (Lark *et al.*, 1963) containing (a) 0.02% (w/v) glucose and (b) 0.002% (w/v) glucose (●) and the lower dilution rates using 0.02% (w/v) glucose (■). Cell volume was determined by means of a Coulter Counter model ZBI/C1000 Channelyzer system linked to an Acorn BBC model B microcomputer.

(Schaechter et al., 1958). Shehata & Marr (1971) reported that the cell volume of E. coli MC30 was directly proportional to growth rate in glucose-limited culture but displayed a more complex response in phosphate-limited culture. E. coli, Paracoccus denitrificans and Bacillus polymyxa when cultured at doubling times of less than approximately 30 hours displayed a direct relationship between growth rate and cell volume but this direct proportionality ceased at doubling times in excess of approximately 33 hours (van Verseveld et al., 1984). It is therefore apparent that although the increase in surface area to volume ratio described in this and other studies may represent a mechanism for increasing relative nutrient uptake ability in these "typical" organisms the nature of the response is not as simple as it may initially appear.

Luscombe & Gray (1974) described the effects of dilution rate in continuous culture on the morphology of Arthrobacter spp., an organism common in the low nutrient natural environment and one that has been proposed as a model oligotroph (Poindexter, 1981a). They cultivated four species in carbon-limited culture and found that below a species-specific dilution rate cells existed as cocci and above this as rods. This rod-sphere transition has been shown not only to function as a means of increasing surface area to volume ratio but enhances survival potential under starvation conditions since the coccoid cells display a greater resistance to starvation than the rods (Luscombe & Gray, 1974). However, the relevance of this transition itself in

nature is open to question since the conditions in the natural soil environment of these isolates is unlikely ever to permit production of rod-shaped cells. Nevertheless, the importance of high surface area to volume ratio in ensuring competitiveness in the natural oligotrophic environment is clear. Matin & Veldkamp (1978) grew a naturally isolated Pseudomonas sp. in continuous culture with a Spirillum sp. from the same environment and showed that the latter successfully outcompeted the former at low growth rates. This success was attributable in part to increased nutrient uptake ability in the Spirillum which itself was partly due to the possession of a higher surface area to volume ratio that was significantly increased at lower dilution rates. Kuenen et al. (1977) observed similar effects when investigating competition for inorganic substrates by bacteria in mixed chemostat cultures. Hoppe (1976) and Torella & Morita (1981) have demonstrated the importance and ubiquity of cell volume reduction in survival and competitiveness in marine bacteria. It is therefore clear that decreased cell volume in morphologically unspecialised bacteria under conditions of nutrient limitation plays a significant role in ensuring competitiveness in oligotrophic environments and that such a response is apparently ubiquitous in bacteria, although the degree of its manifestation is variable.

Whilst in this and many other investigations no study of cell viability at low dilution rates was performed it is known that some organisms exhibit a marked reduction in viability at low growth rates in continuous culture. Tempest

et al. (1967) showed that Enterobacter aerogenes when grown at dilution rates of greater than 0.1h^{-1} was 95-100% viable but at a dilution rate of 0.01h^{-1} viability was decreased to 65%. It was proposed that this was indicative of the existence of a minimum growth rate (μ_{\min}) value specific to this organism. Luscombe & Gray (1974) demonstrated that there was no apparent reduction in population viability of Arthrobacter at a dilution rate of 0.01h^{-1} . Unfortunately, little information is available concerning viability of other bacteria characteristic of oligotrophic environments in continuous culture. However, it is probable that significant variations in values will be observed and data for bacteria from oligotrophic environments should prove most interesting.

3.2.7) Carbon-limited continuous culture of *Hyphomicrobium* X
Hyphomicrobium X was chosen for continuous culture studies on the responses of prosthecate bacteria to growth rate because of the ease with which it can be cultured and the minimal occurrence of wall growth. The organism was cultivated aerobically at 30°C in HM medium containing 100mM methylamine and 10mM phosphate and was shown to be carbon-limited by increasing the concentration of methylamine in the medium reservoir to 150mM and observing a consequent increase in cell numbers in the culture vessel. The μ_{\max} of this organism under these conditions was 0.054h^{-1} (maximum t_d 12.9 h) which was similar to that obtained in batch culture (described above and previously reported;

Attwood & Harder, 1977). Decreasing dilution rate resulted in a slight decrease in the peak cell volume of both the swarmer and prosthecae cells and an increase in prostheca length (Fig. 3.37a). Indeed, the elongation of the prosthecae at low dilution rates may have masked to an extent the actual reduction of the main cell body of the prosthecae cell. These two factors together resulted in a marked increase in the surface area to volume ratio of this organism with increasing carbon stress. The relative proportions of reproducing prosthecae cells, non-reproducing prosthecae cells and swarmer cells also altered with dilution rate (Fig. 3.37b). At low dilution rates the ratio of swarmer cells to non-reproducing prosthecae cells was at its highest and that of reproducing to non-reproducing prosthecae cells lowest. The situation was reversed at higher dilution rates.

Whilst it is probable that these investigations may have slightly underestimated the true length of the prosthecae due to shrinkage during specimen preparation for electron microscopy (Montesinos et al., 1983), the net effect observed is clear. Harder & Attwood (1978) reported that prostheca length of Hyphomicrobium X in methanol-limited continuous culture increased with decreasing dilution rates and that the volume of the main cell body was reduced. The increase in prostheca length as a function of growth rate in carbon-limited, phosphate-excess media once again casts doubt on the suggestion of Poindexter (1984a; 1984b) that the prosthecae primarily function as sites of phosphate uptake. If phosphate concentration was the main

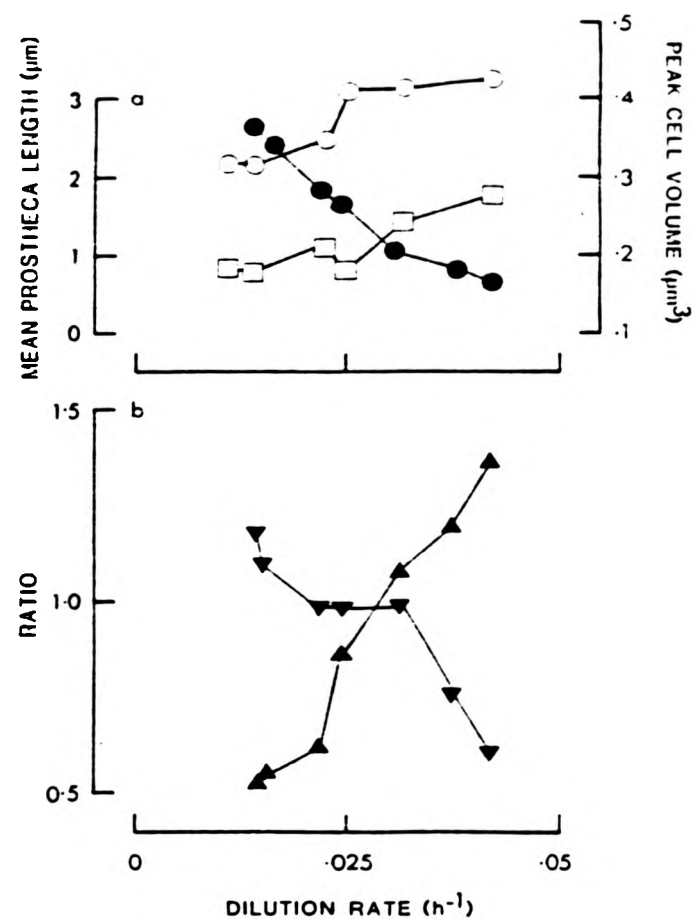


FIGURE 3.37 - Cell type expression, cell volume and prostheca length of *Hyphomicrobium X* in carbon-limited continuous culture. Cultures were grown to steady state at 30°C in HM medium (Attwood & Harder, 1972) containing 100mM methylamine and 10mM phosphate. The upper graph (a) illustrates mean prostheca length (●) as determined by direct measurements of in excess of 100 cells in electron microscope preparations and peak cell volumes of prosthecae cells (○) and swarmer cells (□) as determined by means of a Coulter Counter model ZBI/C1000 Channelyzer system linked to an Acorn BBC model B microcomputer. The lower graph (b) illustrates the ratios of reproducing cells to non-reproducing prosthecae cells (▲) and swarmer cells to non-reproducing prosthecae cells (▼) as determined by direct cell type counts of in excess of 300 cells in electron microscope preparations.

factor controlling prostheca length it is unlikely that prosthecae would elongate with decreasing growth rate due to carbon-limitation alone.

The preferential expression of swarmer cells at low dilution rates is evidence in support of the model of Dow et al. (1983) which proposed that swarmer cells function as metabolically quiescent survival and dispersal stages and that their accumulation at low dilution rates is indicative of an environmental inhibition of differentiation. If these cells do indeed represent metabolically shut-down stages then it is clear that this will have marked consequences on the calculation of growth parameters in continuous culture studies. Tempest (1970) discussed the consequences of the reduced viability of some organisms at low dilution rates on these calculations and stated that if a proportion of the population was inactive then dilution rate (D) is no longer equal to the specific growth rate (μ) nor directly related to doubling time (t_d). Thus the classical basic equations of continuous culture no longer hold true under such conditions and it is necessary to take into account the proportion of viable cells in the population. Tempest (1970) proposed the use of a viability index (α) in such cases:

$$\alpha = (V+1)/2$$

where V is the fraction of viable cells. The basic equations of continuous culture can then be modified to account for the viability index, e.g.:

$$t_d^* = (\ln 2 \cdot \alpha) / D$$

and
$$\mu^* = \ln 2 / t_d^* = (D \cdot \ln 2) / (\ln 2 \cdot \alpha)$$

where $\bar{}$ designates the corrected values obtained. By applying these equations to the data obtained by Tempest et al. (1967) it has been shown (Tempest, 1970) that the Enterobacter aerogenes grown at a dilution rate of 0.004h^{-1} and an apparent t_d of 173h in fact corresponded to a viable fraction t_d^* of 80h. If the swarmer cells of the prosthecate, and potentially other, bacteria are indeed metabolically quiescent then once again the basic equations of continuous culture will be inadequate and lead to an underestimation of growth rate. However, not only will the mathematical modelling of a transiently partially physiologically repressed sub-population itself present difficulties but at present nothing is known regarding the control or metabolic activity of the "swarmer cell state". If such observations as those of van Verseveld et al. (1984) and Koch (1979) prove to be indicative of a widespread existence of dormant swarmer-type cells in slowly growing populations of morphologically "typical" bacteria, then such considerations will have a wider application than may be immediately apparent at present.

3.2.B) Phosphate-limited continuous culture of

Hyphomicrobium X

This organism was cultivated in HM medium containing 100mM methylamine and 1mM phosphate and was shown to be phosphate-limited by increasing the concentration of phosphate in the medium reservoir to 2mM and observing a consequent increase in cell numbers in the culture vessel.

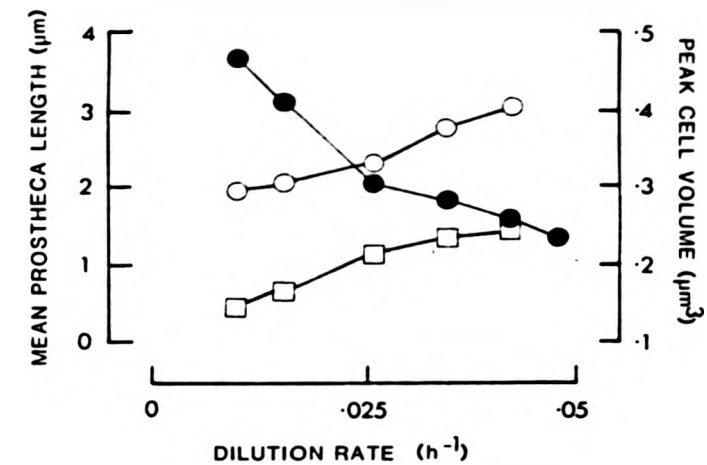


FIGURE 3.38 - Cell volume and prostheca length of *Hyphomicrobium* X in phosphate-limited continuous culture. Cultures were grown to steady state at 30°C in HM medium (Attwood & Harder, 1972) containing 100mM methylamine and 1mM phosphate. The graph illustrates mean prostheca length (●) as determined by direct measurements of in excess of 100 cells in electron microscope preparations and peak cell volumes of prosthecate cells (○) and swarmer cells (□) as determined by means of a Coulter Counter model ZBI/C1000 Channelyzer system linked to an Acorn BBC model B microcomputer.

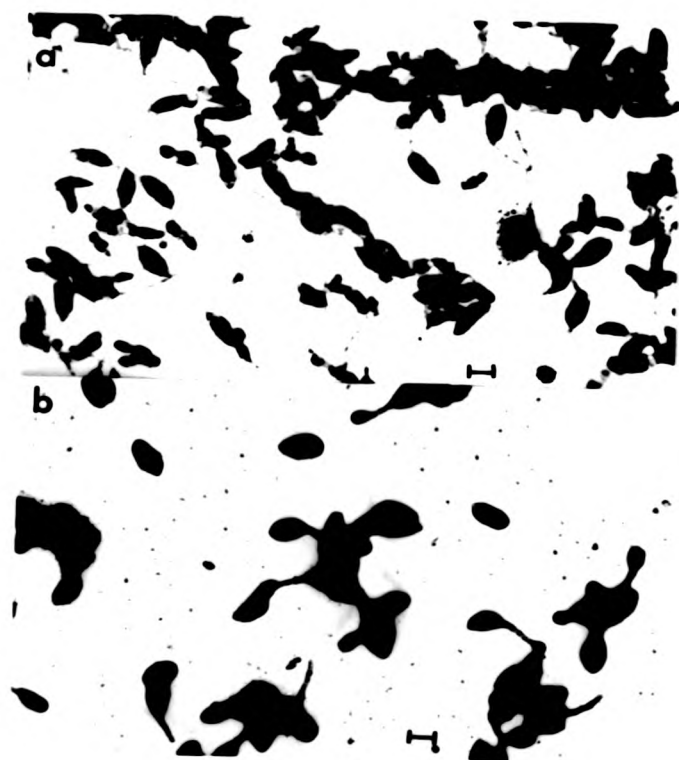


FIGURE 3.39 - Morphology of Hyphomicrobium X in phosphate-limited continuous culture. Cultures were grown to steady state in HM (Attwood & Harder, 1972) containing 100mM methylamine and 1mM phosphate. (a) Cells obtained at a dilution rate of $0.015h^{-1}$. (b) Cells obtained at a dilution rate of $0.048h^{-1}$. Electron micrographs of phosphotungstic acid stained specimens. Scale bar = $1.0\mu m$.



FIGURE 3.39 - Morphology of *Hyphomicrobium* X in phosphate-limited continuous culture. Cultures were grown to steady state in HM (Attwood & Harder, 1972) containing 100mM methylamine and 1mM phosphate. (a) Cells obtained at a dilution rate of $0.015h^{-1}$. (b) Cells obtained at a dilution rate of $0.048h^{-1}$. Electron micrographs of phosphotungstic acid stained specimens. Scale bar = $1.0\mu m$.

The μ_{max} in this medium as determined by the washout rate method was $0.050h^{-1}$ (maximum t_d 13.8h). As was observed in carbon-limited culture, decreasing dilution rate resulted in an increase in prostheca length and a decrease in the cell volume of both prosthecae and swarmer cells (Figs. 3.38 and 3.39). The increase in cell surface area to volume ratio appeared to be somewhat more marked than that observed in carbon-limited culture which suggests that phosphate-limitation has a more significant effect on cellular morphology than carbon-limitation. However, it is clear that in both laboratory culture and the natural environment the control of the cellular morphology of prosthecae bacteria is not controlled solely by the levels of one nutrient but is a function of the pertinent growth-limiting factor or factors. Further investigations are necessary in order to detail these responses and such data should significantly increase our understanding of the nature of the adaptations of prosthecae bacteria to the natural low nutrient environment.

3.2.9 The responses of prosthecae bacteria to their environments

In this study I have chosen to concentrate on two of the basic characteristics of prosthecae budding bacteria which are presumed to play an important role in ensuring survival and competitiveness in the natural environment, namely the production of swarmer cells and prosthecae elongation, both of which are easily quantifiable but neither of which have previously been quantitatively studied in detail. All three

organisms tested displayed elongation of prosthecae and decrease in the volume of the cell body with decreasing nutrient concentration which consequently significantly increase their surface area to volume ratios under such conditions. It is further evident, both in batch culture and particularly in continuous culture of Hyphomicrobium X, that both limiting phosphate and organic carbon concentrations have a significant effect. These observations, as discussed above, are by no means entirely novel but, when considered together, suggest that the model of Poindexter (1984a; 1984b) which proposed that the prosthecae function primarily as the main phosphate uptake site for the cell is unsound. This is not to say that prosthecae do not play a highly significant role in phosphate acquisition in these organisms but that the responses of these organisms to nutrient limitation are more complex than can readily be studied in simple laboratory culture. Further, more detailed, investigations on nutrient effects on cell surface area and the transport of nutrients by prosthecae bacteria are necessary before the questions concerning the role of the prosthecae in nutrient capture can be answered. It is probable that, even then, their potential importance will be underemphasised unless consideration is given to their other potential roles in intermediary metabolism, flotation, reproduction and attachment.

The importance of swarmer cells is similarly unclear. Whilst it has been shown that they accumulate and are apparently maintained under conditions of nutrient stress,

there is a relative dearth of information on their physiology and ecology (Dow et al., 1983; Morgan & Dow, 1985). Of particular importance are the mechanisms of cell type control and environmental sensing and information concerning their distribution in oligotrophic environments containing prosthecate bacteria.

This work has illustrated that prosthecate budding bacteria respond morphologically to the trophic status of their environment and it is to be expected that these adaptations may have profound but "invisible" metabolic counterparts which are as yet unstudied. In this respect the prosthecate bacteria can be considered as "model" oligotrophic bacteria but only in the sense that they are easily manipulated and examined laboratory organisms. As such they can provide an insight into the nature of the bacteria characteristic of oligotrophic environments but only if the results obtained are put into the correct perspective with respect to the potential conditions in situ.

3.2.10) Behaviour of prosthecate bacteria in the natural environment

I have repeatedly stated that many of the studies of bacteria characteristic of low nutrient environments are naive in that they make no attempt to relate their observations to what might occur in the natural environment. Indeed, this investigation in its use of batch culture, well stirred continuous culture and long-established laboratory strains itself falls to an extent into the same trap. Whilst the

conditions employed in this study by no means attempt to model the physical situation in the natural environment, it must be stated, in defence of this approach, that the provision of basic background information is a necessary prerequisite for understanding investigations performed under more realistic conditions.

The prosthecae bacteria characteristic of oligotrophic environments normally possess elongated prosthecae and there is some evidence that swarmer cells are present (see Section 3.1). The probable role of the former in increasing surface area to volume ratio in situ has been borne out by laboratory investigation as has the potential of the latter as a survival and dispersal cell (Dow et al., 1983). In the natural environment, with the exception of Ancalomicrobium and related forms, the majority of prosthecae bacteria would be expected to be found at interfaces where the bulk of bacterial activity in the oligotrophic aquatic environment is likely to occur (Fletcher & Marshall, 1982). Both prosthecae and swarmer cells may also play direct roles under such conditions, the former sometimes in attachment, and possibly as a means of raising the cell body or the daughter cell away from the surface (Aristovskaya, 1963), and the latter as a propagation stage.

Once again it is clear that the information available concerning the ecology and physiology of the prosthecae bacteria in the oligotrophic natural environment is inadequate for understanding the adaptations of all bacteria for an oligotrophic existence. Also, few data are available

on the responses of these organisms, in laboratory culture or the natural environment, to temporal alterations in nutrient flux. It is clear that understanding the behaviour of "oligotrophic" bacteria requires a great deal of further study. However, this investigation has clearly demonstrated that the production of swarmer cells and increased surface area are both important adaptive features in prosthecate bacteria both in the natural environment and in laboratory culture. Furthermore, these responses may well occur in most bacteria growing under conditions of nutrient limitation and the prosthecate bacteria present unrivalled opportunities for their study.

3.3) PHYSIOLOGICAL STUDIES

In order to investigate some of the potential physiological adaptations of swarmer cells, Rhodospirillum rubrum Rm5 was employed and some of the enzymes characteristic of both biosynthetic and catabolic metabolic processes investigated. This organism was chosen because of the ease with which it can be synchronised, making it an ideal tool for investigating differential metabolic activities in the swarmer and prosthecate cells, and because, unlike many of the other members of the Rhodospirillaceae, there is little information available concerning its metabolism.

3.3.1) Activity and inhibitor patterns of tricarboxylic acid

cycle enzymes of *Rhodospirillum rubrum*

The tricarboxylic acid (TCA) cycle can be considered as having two functions, it is the most common pathway involved in the oxidation of organic compounds and it is directly involved in the synthesis of biosynthetic intermediates (Weitzman, 1982). The cycle can be considered as consisting of four distinct stages (Fig. 3.40). Stage (a) involves the entry of two carbon units into the cycle in the form of acetate by means of the condensation of acetyl-CoA with oxaloacetate to produce citrate. This is followed by two successive decarboxylation stages (b and c) which ultimately result in the production of a four carbon unit, succinyl-CoA via 2-oxoglutarate. In bacteria these two steps normally result in the production of NADPH and NADH respectively. In a sense the sequence of reactions have achieved their goals: they have oxidised two carbon atoms, generated energy and produced the key biosynthetic intermediates 2-oxoglutarate and succinyl-CoA. However, the final stage of the cycle (d), the conversion of succinyl-CoA to oxaloacetate, results in regeneration of carrier molecules for incorporation of subsequent acetate units. This cycle of reactions is most commonly altered in bacteria by the absence of 2-oxoglutarate dehydrogenase which prevents the production of succinyl-CoA and necessitates the production of this compound by other means. Whilst this commonly occurs in a variety of autotrophic bacteria (Smith & Hoare, 1977), the members of the Rhodospirillaceae examined to date possess complete TCA

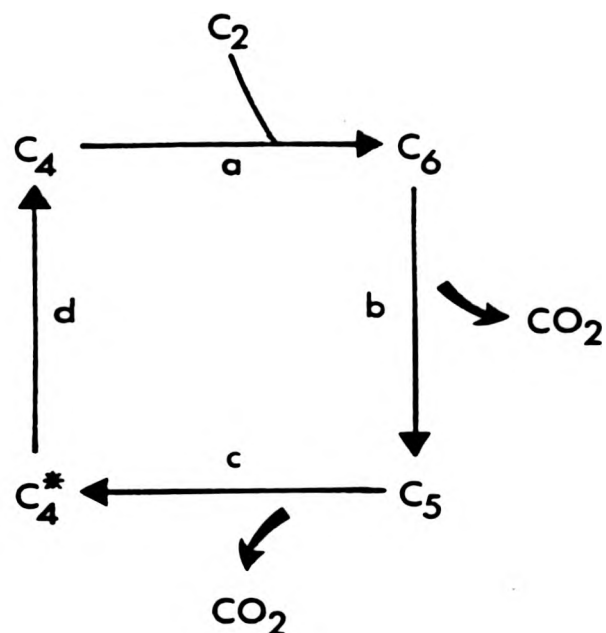


FIGURE 3.40 - Diagrammatic representation of the TCA cycle. Stage a illustrates the entry of carbon as acetyl units (C_2) involving their condensation with C_4 units to form C_6 units. There follow two oxidative decarboxylation phases (b and c), involving CO_2 liberation and, in bacteria, the production of NADPH and NADH respectively, which ultimately result in the production of succinyl-CoA (C_4^*). The remaining phase of the cycle (d) is concerned with the regeneration of the original C_4 units for condensation with acetyl-CoA. After Weitzman (1982).

cycles (Beatty & Gest, 1981a; 1981b). In view of the importance of the TCA cycle in the provision of biosynthetic intermediates, the enzymes of this pathway were a logical choice for investigation of the physiology of swarmer and prosthecate cells of R. vannielii.

R. vannielii was readily disrupted by the use of the French pressure cell and by ultrasonication (Fig. 3.41), although for reasons of convenience the latter was the method routinely employed. Similarly convenient and successful was the preparation of homogeneous populations of swarmer cells by the filtration method of Whittenbury & Dow (1977). Figure 3.42 illustrates an early stationary phase culture of R. vannielii before and after synchronisation. The process was quantified by electronic particle size analysis using the Coulter Counter (Fig. 3.43), where distinct cell volume peaks corresponding to swarmer and prosthecate cells could be detected in heterogeneous cultures and solely the former following synchronisation.

The activities of all of the enzymes of the TCA cycle were analysed in photoheterotrophically grown cells and the results are illustrated in Table 3.4. No differences in activity were detected between lysates prepared by the two methods of cell disruption. All enzymes of the cycle were present at equivalent activities in both heterogeneous and swarmer cell populations with the exception of 2-oxoglutarate dehydrogenase which was apparently absent from both cell types. All enzymes present exhibited comparable activities except for malate dehydrogenase which was far more active

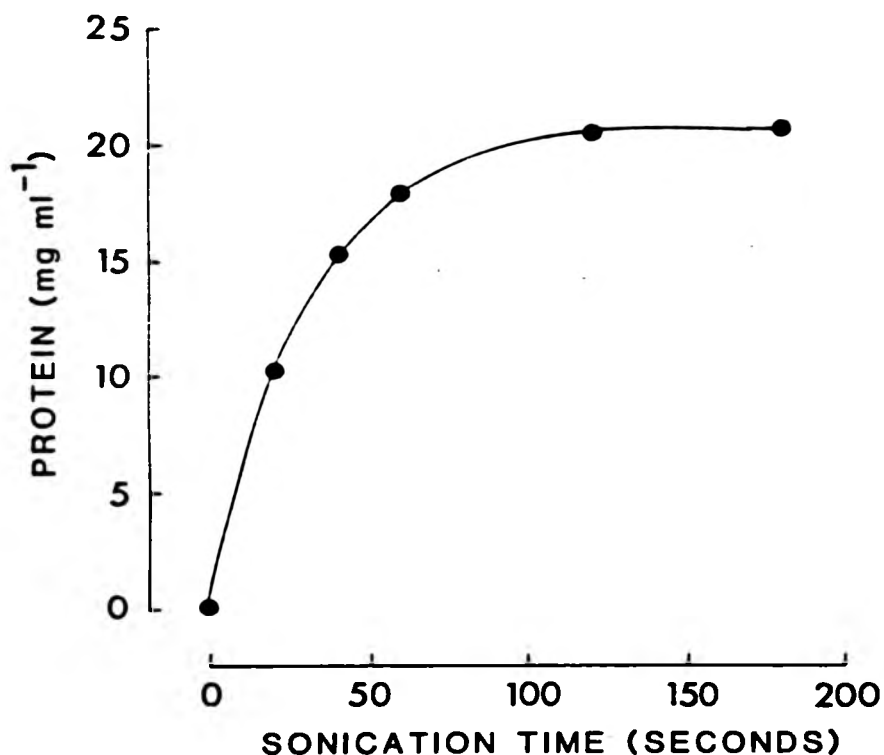


FIGURE 3.41 - Disruption of Rhodomicrobium vannielii by sonication. A cell paste of Rhodomicrobium vannielii was disrupted by ultrasonication as described in the text and samples taken at intervals. Undisrupted cells and cell debris were removed by centrifugation at 4°C for 10 minutes in an Eppendorf microfuge and the protein concentration in the supernatant determined using the method of Lowry et al. (1951).



FIGURE 3.42 - Heterogeneous and synchronous populations of *Rhodospirillum rubrum*. (a) Early exponential phase culture prior to synchronisation. (b) Swarmer cell population produced following synchronisation using the filtration method of Whittenbury & Dow (1977). Electron micrographs of phosphotungstic acid stained specimens. Scale bar = 1.0µm.



FIGURE 3.42 - Heterogeneous and synchronous populations of *Rhodospirillum rubrum*. (a) Early exponential phase culture prior to synchronisation. (b) Swarmer cell population produced following synchronisation using the filtration method of Whittenbury & Dow (1977). Electron micrographs of phosphotungstic acid stained specimens. Scale bar = 1.0µm.

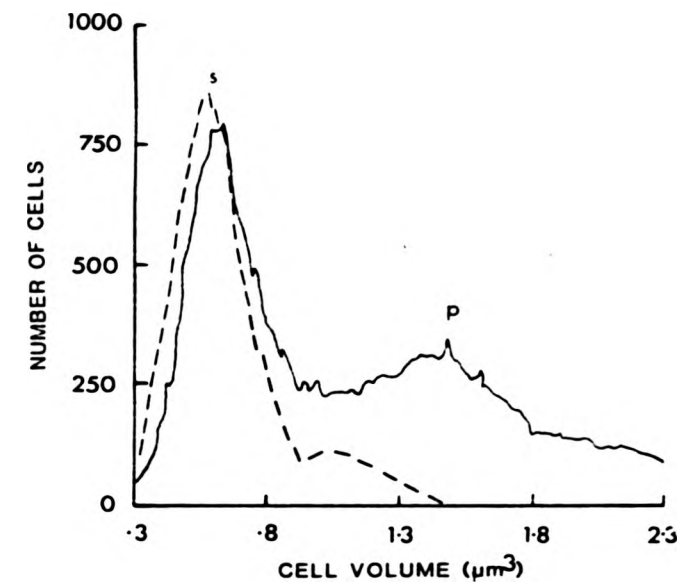


FIGURE 3.43 - Synchronisation of *Rhodospirillum rubrum* illustrated by electronic particle size analysis. An early stationary phase culture (solid line) and the swarmer cell population resulting from its synchronisation using the method of Whittenbury & Dow (1977) (broken line) were diluted in Isoton and analysed by means of a Coulter Counter model ZBI (operating at a 1/amplification of 0.5) linked to a C1000 Channelyzer system and an Acorn BBC model B microcomputer. s, swarmer cell peak; p, prosthecae cell peak. Chains of cells are not resolved using the Coulter Counter at this amplification but were not present in the synchronous population (Fig. 3.42).

TABLE 3.4 - Activities of TCA cycle enzymes in *Rhodocrobium vanniellii* cell-free extracts. Enzymes were assayed as described above (Section 2.6.3) at room temperature using lysates prepared from heterogeneous and swarmer cell populations. Values presented are the means of at least three independent experiments with at least three assays performed each time and are corrected for background activity. ND = not determined. dh = dehydrogenase.

<u>Enzyme</u>	<u>EC no.</u>	<u>Activity (nmol min⁻¹ (mg protein)⁻¹)</u>	
		<u>Heterogeneous</u>	<u>Swarmer cells</u>
citrate synthase	4.1.3.7	37.1	45.2
aconitase	4.2.1.3	43.3	40.0
isocitrate dh			
NADP-linked	1.1.1.42	242.1	229.6
NAD-linked	1.1.1.41	74.5	59.2
2-oxoglutarate dh system		<0.1	<0.1
succinate thiokinase	6.2.1.4	23.6	25.3
succinate dh	1.3.99.1	75.7	ND
funarase	4.2.1.2	10.8	ND
malate dh	1.1.1.37	1835.0	1821.0

than the other TCA cycle enzymes. In all cases dependence of reaction on substrate and linearity between protein concentration and reaction rate were demonstrated.

Three reactions in particular were deemed worthy of further investigation. The activity of aconitase presented is that observed using *cis*-aconitate as a substrate. However, the enzyme reaction occurs in two stages, the first being the conversion of citrate to *cis*-aconitate. When the enzyme was assayed using citrate as the substrate the activity obtained was decreased by a factor of approximately ten (Figure 3.44;

observed activity $4.2 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$). This pattern of aconitase activity on the two distinct substrates is to be expected since the equilibrium position of the citrate to aconitate reaction tends very strongly towards citrate (Reeves *et al.*, 1971). The possession of both NAD and NADP-linked isocitrate dehydrogenase activity is comparatively unusual in bacteria (Weitzman, 1982). In this case the activity observed for the characteristically bacterial NADP-linked enzyme was consistently three to four times higher than that of the NAD-linked enzyme (Table 3.4; Fig. 3.45). The detection of 2-oxoglutarate dehydrogenase activity in cell lysates is notoriously difficult (Reeves *et al.*, 1971), however, activity was readily detectable using lysates of aerobically grown *E. coli* and also using photoheterotrophically grown lysates of *Rhodopseudomonas blautica* (Fig. 3.46) and subsequent experiments demonstrated that cell-free lysates prepared from cultures of *R. vannielii* grown under dark aerobic (chemoheterotrophic) conditions possessed this enzyme (Fig. 3.46) at an activity of $43.2 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$. These data suggest that cells of *R. vannielii* grown anaerobically possess a complement of TCA cycle enzymes that is complete except for 2-oxoglutarate dehydrogenase and that this enzyme is produced under aerobic conditions.

Whilst crude studies of the activities of enzymes yield some basic data on the nature of the TCA cycle and the control of enzyme synthesis, the regulation of enzyme activity *in vivo* undoubtedly involves a variety of subtle

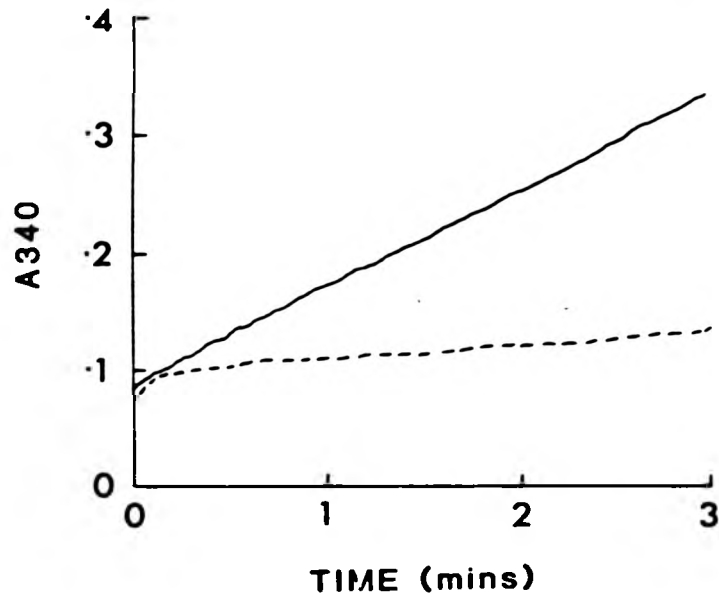


FIGURE 3.44 - Assay of aconitase activity in a cell-free extract of *Rhodomicrobium vannielii*. The assay was performed as described in the text using 0.36 mg protein ml⁻¹ and the reaction followed by recording the substrate-dependent increase in absorbance at 340nm following the addition of aconitate (solid line) or citrate (broken line) by means of a Servoscribe chart recorder linked to a Gilford model 250 spectrophotometer.

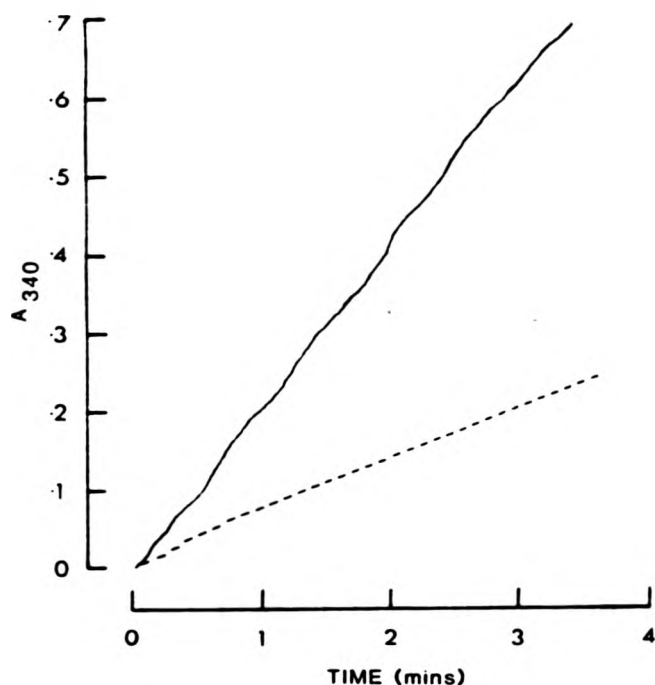


FIGURE 3.45 - Assay of isocitrate dehydrogenase activity in a cell-free extract of *Rhodomicrobium vannielii*. The assay was performed as described in the text using 0.11 mg protein ml⁻¹ and the reaction followed by recording the isocitrate-dependent increase in absorbance at 340nm following the addition of NADP (solid line) or NAD (broken line) by means of a Servoscribe chart recorder linked to a Gilford model 250 spectrophotometer.

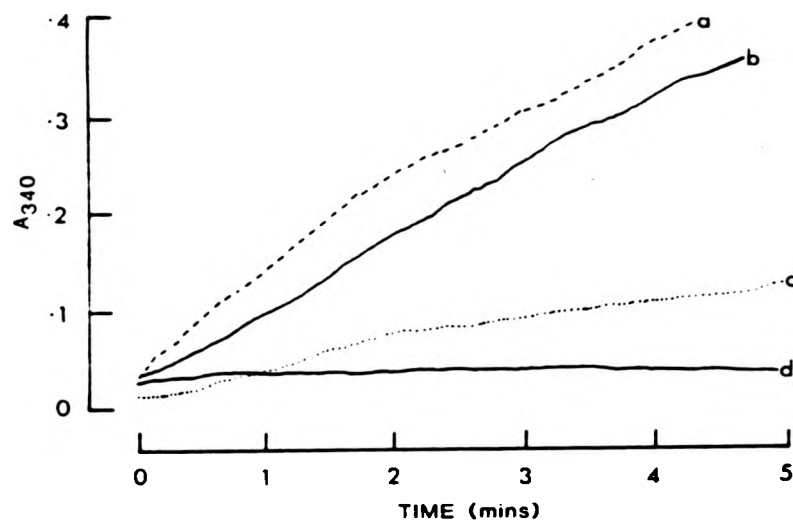


FIGURE 3.46 - Assay of 2-oxoglutarate dehydrogenase activity. The assay was performed as described in the text and the 2-oxoglutarate-dependent reaction measured at 340nm by means of a Gilford model 250 spectrophotometer and recorded using a Servoscribe chart recorder. (a) Lysate of aerobically grown Escherichia coli, 0.30mg protein ml⁻¹. (b) Lysate of aerobic chemoheterotrophically grown Rhodomicrobium vannielii, 0.40mg protein ml⁻¹. (c) Lysate of photoheterotrophically grown Rhodopseudomonas blastica, 0.32mg protein ml⁻¹. (d) Lysate of photoheterotrophically grown Rhodomicrobium vannielii, 0.44mg protein ml⁻¹.

TABLE 3.5 - Inhibitors of *Rhodomicrobium vannielii*

citrate synthase. The enzyme was assayed as described in the text using cell-free extracts prepared from heterogeneous and swarmer cell populations and activities are expressed as a percentage of that obtained in the absence of inhibitor. ND = not determined.

<u>Inhibitor</u>	<u>Concentration (mM)</u>	<u>Activity as % of control</u>	
		<u>Heterogeneous</u>	<u>Swarmer cells</u>
ATP	1.0	98.4	ND
	2.0	92.3	94.3
	5.0	75.0	77.6
	10.0	56.7	53.0
ADP	2.0	98.1	ND
	5.0	97.3	ND
	10.0	88.7	89.6
AMP	10.0	99.0	99.8
NAD	5.0	100.1	100.2
NADH	0.1	92.4	92.4
	0.5	53.7	55.6
	1.0	15.9	11.6
	2.0	10.8	ND
NADH+1mM AMP	1.0	98.4	97.4
	2.0	95.3	ND
2-oxoglutarate	10.0	101.7	103.4

physiological control mechanisms. The most studied TCA cycle enzyme with respect to inhibition is citrate synthase, the control of which, because of its key position in the cycle at the stage of the entry of two carbon units, has profound effects on the whole TCA cycle (Weitzman, 1982). A variety of demonstrated citrate synthase inhibitors were tested for effects on the citrate synthase of *R. vannielii*. The results are shown in Table 3.5 and can be more clearly

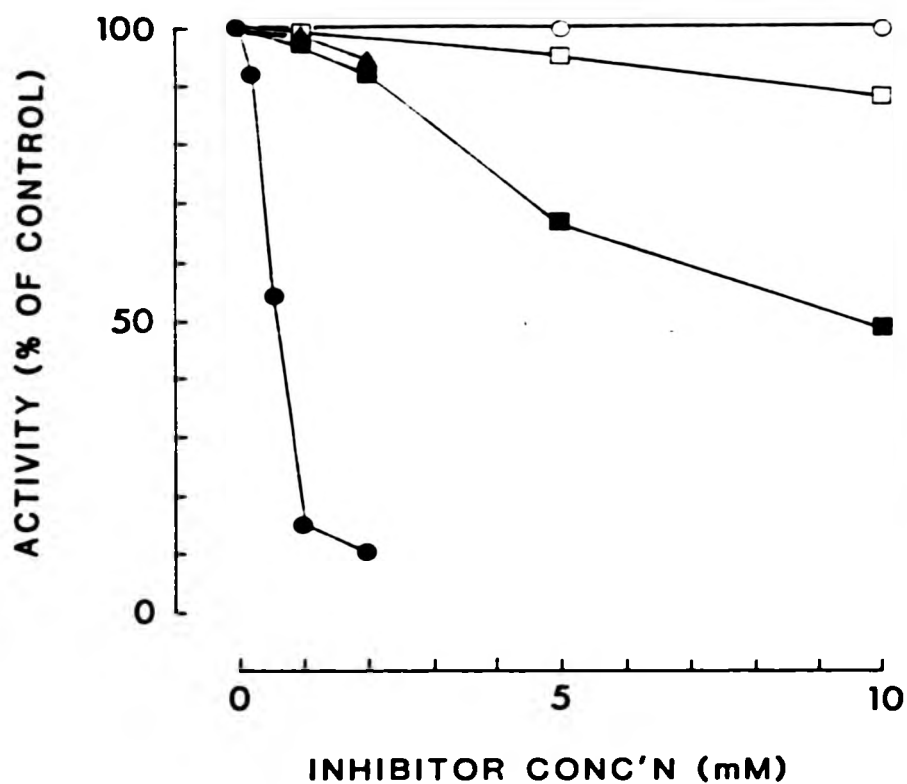


FIGURE 3.47 - Effects of inhibitors on the citrate synthase of *Rhodococcus vannielii*. The graph illustrates the activity of citrate synthase in cell-free extracts of *R. vannielii* heterogeneous cultures in the presence of various potential inhibitors as a percentage of that in their absence. For details see text. (●) NADH. (▲) NADH + 1mM AMP. (■) ATP. (□) ADP. (○) AMP or NAD.

appreciated when illustrated graphically as for the heterogeneous population in Figure 3.47. No significant differences in inhibitor effects between heterogeneous and swarmer cell populations was observed. The enzyme was markedly inhibited by adenosine nucleotides, with ATP having the greatest effect and AMP the smallest. NADH but not NAD greatly inhibited the enzyme and this effect was reversed by the addition of 1mM AMP. 2-oxoglutarate did not reduce enzyme activity even at concentrations as high as 10mM. The significance of these results will be discussed below (Section 3.3.5).

3.3.2) Incorporation of radiolabelled carbon into cellular compounds by *Rhodomicrobium vannielii* and *Rhodopseudomonas palustris*

These investigations were performed in order to confirm the absence of 2-oxoglutarate dehydrogenase from *R. vannielii*. ^{14}C -labelled acetate has been frequently used to demonstrate the absence of this enzyme in bacteria since, in the absence of 2-oxoglutarate dehydrogenase, acetate enters the split TCA cycle in the normal fashion via the action of citrate synthase but the distribution of radiolabel into cellular amino-acids is virtually restricted to those whose biosynthetic pathways originate from intermediates produced in the TCA cycle reactions prior to 2-oxoglutarate dehydrogenase, namely leucine, glutamine, proline and arginine (Smith & Hoare, 1977). If the cycle is complete then radiolabel is distributed more equally throughout the range of cellular amino-acids.

Rhodopseudomonas palustris was employed in these experiments as an organism possessing a complete TCA cycle under growth conditions virtually identical to those used for R. vannielii (Eley et al., 1979). Both organisms were grown in the presence of the [U- ^{14}C]-acetate, [1- ^{14}C]-acetate and [2- ^{14}C]-acetate to demonstrate that incorporation of radiolabel into other amino-acids was not a function of the subsequent incorporation of $^{14}\text{CO}_2$ produced during the conversion of isocitrate to 2-oxoglutarate. In addition, cells were grown in the presence of [U- ^{14}C]-malate which would be expected to yield a uniform distribution of radiolabel since it is an intermediate in the TCA cycle beyond 2-oxoglutarate dehydrogenase.

Preliminary qualitative observations using autoradiography of two-dimensional chromatograms of purified cellular protein hydrolysates (Fig. 3.4B) suggested that R. vannielii incorporated radiolabel primarily into leucine, glutamine, proline and arginine. These observations were quantified for both organisms by liquid scintillation counting of individual amino-acid spots on chromatograms (Table 3.6) and these four amino-acids accounted for 72.0% of the total detected radioactivity in lysates from cells grown in the presence of [U- ^{14}C]-acetate in contrast to 32.6% in the equivalent culture of Rhodopseudomonas palustris. These data also show that the incorporation of radiolabel into amino-acids in R. vannielii is independent of the position of the radiolabelled carbon atom in the acetate

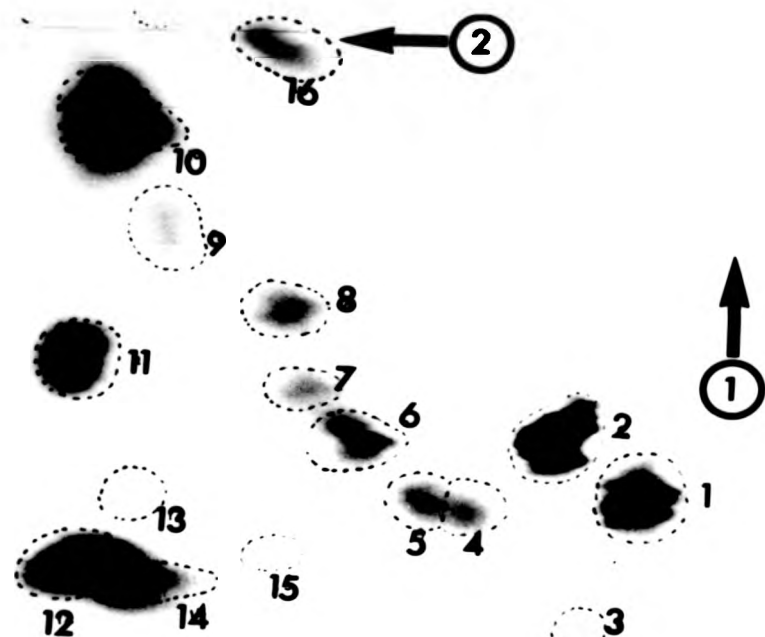


FIGURE 3.48 - Autoradiograph of two-dimensional chromatogram of [U- ^{14}C]-acetate labelled *Rhodospirillum rubrum* protein hydrolysate. The solvent directions are illustrated by the arrows. For details of experimental techniques see text. The positions of the amino-acid spots on the original ninhydrin stained chromatogram are marked: 1, aspartate; 2, glutamine; 3, cysteine, cystine; 4, serine; 5, glycine; 6, threonine; 7, alanine; 8, tyrosine; 9, valine; 10, leucine, iso-leucine, phenylalanine; 11, proline; 12, arginine; 13, methionine; 14, histidine; 15, ornithine; 16, unidentified. Autoradiograph kindly supplied by Dr. C.S. Dow.

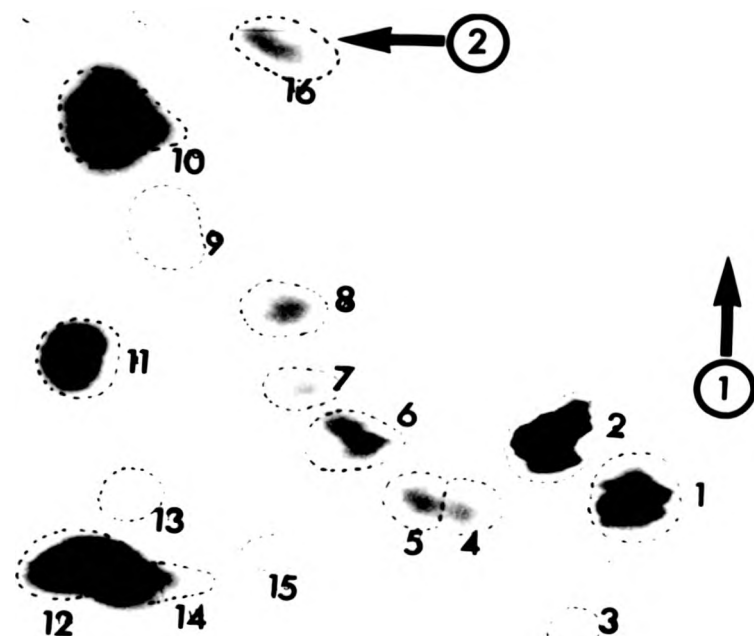


FIGURE 3.48 - Autoradiograph of two-dimensional chromatogram of [U- ^{14}C]-acetate labelled *Rhodospirillum rubrum* protein hydrolysate. The solvent directions are illustrated by the arrows. For details of experimental techniques see text. The positions of the amino-acid spots on the original ninhydrin stained chromatogram are marked: 1, aspartate; 2, glutamine; 3, cysteine, cystine; 4, serine; 5, glycine; 6, threonine; 7, alanine; 8, tyrosine; 9, valine; 10, leucine, iso-leucine, phenylalanine; 11, proline; 12, arginine; 13, methionine; 14, histidine; 15, ornithine; 16, unidentified. Autoradiograph kindly supplied by Dr. C.S. Dow.

TABLE 3.6 - Incorporation of radiolabel from [^{14}C]-acetate and [U- ^{14}C]-malate into cellular amino-acids by *Rhodomicrobium vannielii* and *Rhodopseudomonas palustris*.

The values presented are expressed as a percentage of total calculated radioactivity loaded to a two-dimensional chromatogram and are the means of three experiments. A proportion of the radioactivity remained at the origin. For details of experimental protocol see text. ND = not detected. U, 1 and 2 in the headings refer to [U- ^{14}C]-acetate, [1- ^{14}C]-acetate and [2- ^{14}C]-acetate respectively. In the bottom line of the table "TOTAL" refers to the percentage recovery of the calculated total radioactivity loaded.

amino-acid	<i>R. vannielii</i>				<i>R. palustris</i>	
	acetate		malate		acetate	malate
	U	1	2		U	
aspartate	5.6	6.2	5.0	7.8	13.6	8.9
glutamine	18.5	15.5	12.6	11.7	8.8	10.2
cysteine						
cystine	0.6	ND	1.6	10.7	10.5	5.9
serine	1.6	2.1	3.6	5.2	3.2	5.8
glycine	2.1	3.5	4.2	5.6	6.0	7.3
threonine	3.6	2.7	3.3	7.5	6.3	7.5
alanine	1.8	3.5	2.7	9.5	6.3	4.8
tyrosine	2.4	2.7	3.3	4.7	8.5	8.0
valine	1.5	1.4	2.2	4.1	2.8	4.1

amino-acid	<u>R. vannielii</u>				<u>R. palustris</u>	
	<u>acetate</u>		<u>malate</u>		<u>acetate</u>	<u>malate</u>
	<u>U</u>	<u>1</u>	<u>2</u>		<u>U</u>	
leucine						
iso-leucine						
phenylalanine	24.2	20.8	23.3	7.6	9.2	12.2
proline	11.4	12.4	13.7	4.8	5.3	5.4
arginine	15.4	14.9	14.1	7.2	10.7	7.1
methionine	0.9	4.3	4.1	3.3	4.1	2.9
lysine						
histidine	4.8	3.3	3.5	7.9	2.5	3.6
ornithine	0.4	1.4	0.4	4.1	3.3	3.9
ORIGIN	1.7	2.3	2.0	1.6	3.2	3.0
TOTAL	96.5	97.0	99.6	102.3	104.3	100.6

molecule and there is therefore no evidence for the incorporation of acetate-derived CO_2 into metabolic intermediates in this organism. The limited distribution of radiolabel into amino-acids observed by autoradiography is confirmed. The incorporation of radiolabel into amino-acids from both sources by Rhodopseudomonas palustris and from $[\text{U}-^{14}\text{C}]$ -malate by R. vannielii is more universal in nature. Indeed, the percentage of the total radioactivity detected in the leucine, glutamine, proline and arginine spots in the latter case is only 30.6%. This demonstrates the existence of a complete TCA cycle in the former organism and the general utilisation of medium-derived malate as a source of biosynthetic intermediates by both organisms.

The absence of 2-oxoglutarate dehydrogenase activity in cell-free extracts of photoheterotrophically grown cells

of R. vannielii was directly confirmed by investigating the utilisation of [5-¹⁴C]-2-oxoglutarate. The assay system (described above) was incubated for up to 60 minutes and organic acids separated by paper chromatography. When a lysate of E. coli was employed, incorporation of radiolabel into succinate was observed but there was no apparent conversion of 2-oxoglutarate to this or other organic acids characteristic of the TCA cycle by lysates of R. vannielii (Fig. 3.49).

3.3.3) Glyoxylate shunt enzymes in *Rhodospirillum* *vannielii*

The glyoxylate shunt or bypass can be considered as being a link between the two "halves" of the TCA cycle. The pathway involves two enzymes: isocitrate lyase which converts isocitrate to glyoxylate and malate synthase which converts glyoxylate to malate. The pathway has at least two distinct functions directly related to the TCA cycle. In bacteria growing aerobically on acetate it functions in an anaplerotic role to replenish the supply of TCA cycle intermediates which would otherwise be depleted by biosynthetic reactions due to the two carbon atoms supplied by acetate being oxidised in the complete cycle. In this case there is a net condensation of two acetate molecules to produce one succinate (Packter, 1973). In a number of autotrophic bacteria, in particular cyanobacteria, the glyoxylate shunt serves as a means for maintaining a cyclic TCA-like sequence of reactions in the absence of 2-oxoglutarate dehydrogenase (Weitzman, 1982).

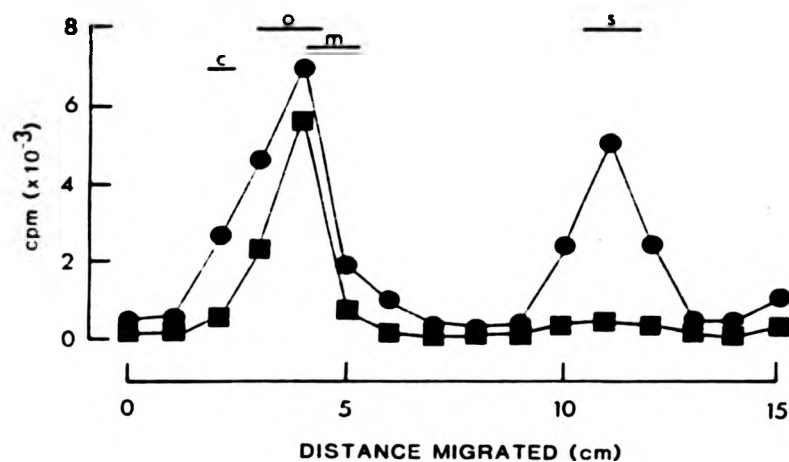


FIGURE 3.49 - Conversion of [5-¹⁴C]-2-oxoglutarate to succinate by cell-free extracts. The assay was performed as described in the text in the presence of 0.1 μ Ci ml⁻¹ potassium [5-¹⁴C]-2-oxoglutarate and portions of the assay mixture subjected to one-dimensional paper chromatography. The chromatograms were cut into 1cm strips in which radioactivity was determined by liquid scintillation counting. (●) *E. coli* lysate, 7.7mg protein ml⁻¹, 30 minute incubation period. (■) Photoheterotrophically grown *R. vannielii* lysate, 7.9mg protein ml⁻¹, 30 minute incubation period. The positions of organic acids on standard chromatograms are illustrated by the bars at the top of the figure: c, citrate; o, 2-oxoglutarate; m, malate; s, succinate.

Isocitrate lyase activity was not detected in lysates of photoheterotrophically grown R. vannielii. However, it has been shown that in many cases expression of glyoxylate shunt enzymes only occurs in the presence of acetate (Packter, 1973) and it was possible that this was occurring in R. vannielii. Activity was not detectable in this organism, however, when cultivated on media containing pyruvate and acetate or acetate alone but could be detected in lysates of Pseudomonas putida grown on acetate. Malate synthase activity was found in lysates of cells grown in all media but activity in cells grown in standard PM medium ($3.36\text{nmol min}^{-1} (\text{mg protein})^{-1}$) was significantly lower than that detected in cells grown in acetate medium ($11.24\text{nmol min}^{-1} (\text{mg protein})^{-1}$).

3.3.4) NADH oxidase activity in *Rhodospirillum rubrum*

This enzyme was originally assayed because its activity directly interferes with those enzyme assays which are measured by following the production or utilisation of NADH. Whilst activity could be detected in E. coli and aerobically grown R. vannielii no enzymic activity could be detected in cells of the latter organism grown photoheterotrophically (Fig. 3.50).

3.3.5) The nature and role of the tricarboxylic acid cycle and related reactions in *Rhodospirillum rubrum*

Whittenbury & Dow (1977) demonstrated that R. vannielii was capable of using all intermediates in the TCA cycle as carbon

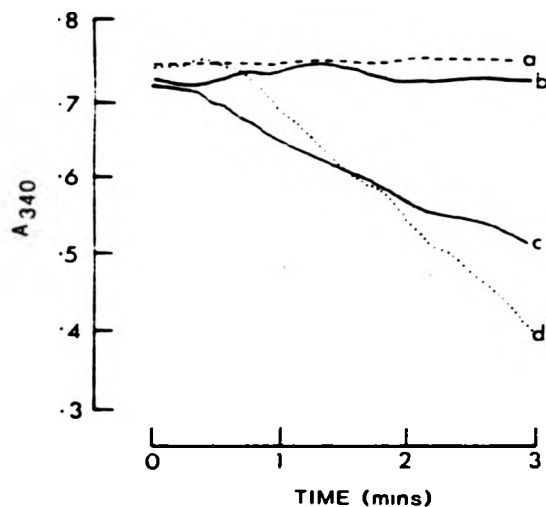


FIGURE 3.50 - Assay of NADH oxidase activity. The assay was performed as described in the text and the NADH-dependent reaction measured at 340nm by means of a Gilford model 250 spectrophotometer and recorded using a Servoscribe chart recorder. (a) Blank cuvette, no lysate. (b) Lysate of photoheterotrophically grown *R. vannielii*, 0.75mg protein ml⁻¹. (c) Lysate of aerobically grown *R. vannielii*, 0.72mg protein ml⁻¹. (d) Lysate of *E. coli*, 0.62mg protein ml⁻¹.

sources with the exception of citrate and 2-oxoglutarate. Whilst data of this kind are determined not only by the intermediary metabolism of the organism under test but by considerations of permeability, this preliminary data suggested that R. vannielii possessed at least some of the enzymes involved in the TCA cycle. The pattern of radiolabel incorporation into amino-acids produced when cells of R. vannielii were labelled with ^{14}C -acetate demonstrated a predominance of incorporation of acetate carbon in leucine, glutamine, proline and arginine. These data were similar to those obtained in a variety of investigations which in all cases were indicative of the operation of an incomplete TCA cycle lacking 2-oxoglutarate dehydrogenase (Smith et al., 1967; Hoare et al., 1967; Smith & Hoare, 1977). The pathways of amino-acid biosynthesis in the Rhodospirillaceae are not atypical (Datta, 1978) and the patterns of radiolabel incorporation therefore suggested the existence of such a split cycle in R. vannielii. A more direct demonstration of the absence of this key enzyme was obtained by the use of radiolabelled 2-oxoglutarate (Pearce et al., 1969), a result which contrasted with those obtained by Chernyadev et al. (1970) who studied the incorporation of acetate carbon into TCA cycle intermediates by Rhodopseudomonas palustris and confirmed the amino-acid labelling patterns described above.

The assays of enzyme activity demonstrated the existence of all TCA cycle enzymes except 2-oxoglutarate dehydrogenase, mostly at approximately comparable activities. However, malate dehydrogenase was an exception, the

exceptionally high activity of which under photoheterotrophic conditions is presumably a function of the utilisation of medium-derived malate for the production of other biosynthetic intermediates. The lack of 2-oxoglutarate dehydrogenase activity, even though the cell-free extracts contained sufficient quantities of membranes to supply adequate levels of this membrane-bound enzyme were it present (Beatty & Gest, 1981b), therefore provides a third line of evidence in support of the existence of an incomplete TCA cycle in R. vannielii. On the basis of the available evidence it appears that R. vannielii has a pattern of TCA cycle-related metabolism similar to that illustrated in Figure 3.51. The right hand branch of the TCA cycle appears to function normally with the incorporation of acetate carbon into the glutamate-derived amino-acids glutamine, arginine and proline. The biosynthetic pathway involved in the production of the other amino-acid that was shown to incorporate a significant quantity of acetate-derived carbon, leucine, is unclear but appears to be via pyruvate or acetyl-CoA (Datta, 1978). The pathways resulting in the production of alanine and valine are similarly unclear but appear to derive from pyruvate, the provision of which in the medium would prevent significant incorporation of acetate-derived carbon into these amino acids. The lack of both 2-oxoglutarate dehydrogenase and a functional glyoxylate shunt means that there is an apparently complete separation of the two halves of the cycle. The radiolabelling patterns show that there is little incorporation of acetate carbon

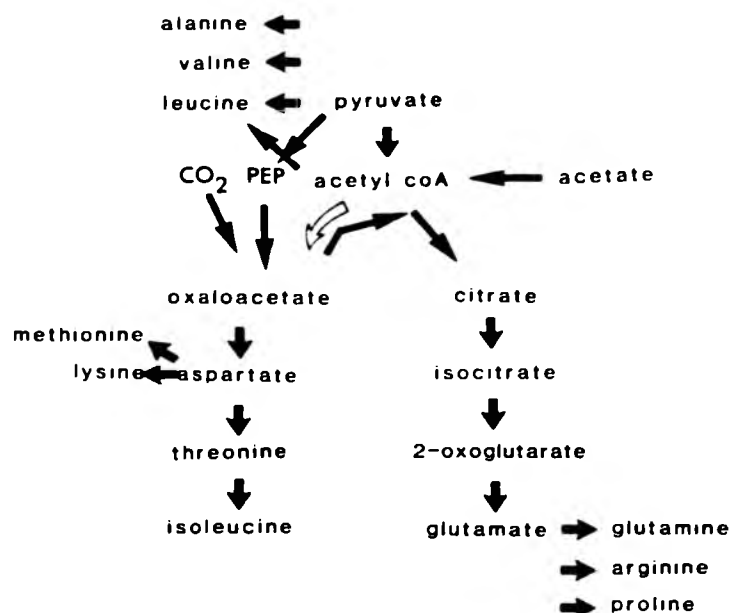


FIGURE 3.51 - Possible TCA cycle-related metabolic pathways operating in photoheterotrophically grown Rhodomicrobium vannielii. The type of system indicated by labelling patterns and direct enzyme assays is based on a split tricarboxylic acid cycle with no incorporation of acetyl-CoA into oxaloacetate (open arrow). For details see text.

into amino-acids synthesised from intermediates produced in the left hand branch of the cycle, suggesting that there is no conversion of acetyl-CoA to oxaloacetate (open arrow) and only minimal entry of carbon from acetate via pyruvate or from acetate-derived CO₂ catalysed by phosphoenolpyruvate carboxylase. The left hand branch of the cycle has been shown to function in many anaerobes as an electron sink (Gest, 1981). The assay of the key enzyme of this process, fumarate reductase, which functions in place of succinate dehydrogenase acting in the reverse direction, was not performed. Whilst it is likely that R. vannielii is producing the bulk of the intermediates necessary for this half of the cycle from medium-derived malate, there is no apparent prima facie evidence for a requirement for this particular enzyme in this organism and evidence from both enzyme assays and the universal incorporation of carbon from malate into amino-acids suggest the presence of succinate dehydrogenase.

Under aerobic conditions 2-oxoglutarate dehydrogenase activity was detected suggesting the operation of a complete oxidative TCA cycle under these conditions. The absence of 2-oxoglutarate dehydrogenase in R. vannielii grown photoheterotrophically is in contrast to the situation found in other members of the Rhodospirillaceae. The existence of a complete TCA cycle, including a low but detectable level of 2-oxoglutarate dehydrogenase activity, under anaerobic photoheterotrophic conditions has been demonstrated by a variety of means in Rhodospirillum rubrum

(Elsden & Ormerod, 1956; Gest et al., 1962; Eisenberg, 1953; Anderson & Fuller, 1967), Rhodopseudomonas palustris (Eley et al., 1979), Rhodobacter capsulatus (Beatty & Gest, 1981a), Rhodocyclus gelatinosus, Rhodobacter sphaeroides, Rhodopseudomonas viridis and Rhodocyclus tenuis (Beatty & Gest, 1981b). In all of these cases activity of 2-oxoglutarate is increased by even very low partial pressures of oxygen (Cox et al., 1983; Beatty & Gest, 1981b). Thus Rhodomicrobium vannielii appears to display a more stringent repression of this enzyme and, under the conditions employed for cultivation, produce succinyl-CoA from malate by a pathway involving the left hand branch of the TCA cycle as has been proposed for Rhodocyclus gelatinosus (Beatty & Gest, 1981b). Investigations of the activity of fumarate reductase and other enzymes of the TCA cycle in R. vannielii under different growth conditions should prove most enlightening. However, whilst the behaviour of R. vannielii is anomalous with respect to the other members of the Rhodospirillaceae, the presence of an incomplete TCA cycle under anaerobic or autotrophic conditions is common. The absence of 2-oxoglutarate dehydrogenase occurs in many thiobacilli (Tabita & Lundgren, 1970; Peeters et al., 1970), methanotrophs (Zhao & Hanson, 1984), cyanobacteria (Pearce et al., 1969; Hoare et al., 1967; Smith et al., 1967) and some methylotrophs (Smith & Hoare, 1977). The position observed in R. vannielii most closely resembles that observed in E. coli (Amarasingham & Davis, 1965), Bacillus polymyxa and B. macerans (Tanaka &

Hanson, 1975) which possess 2-oxoglutarate dehydrogenase activity under aerobic but not anaerobic conditions.

Also of interest in R. vannielii is the possession of both NAD and NADP-linked isocitrate dehydrogenases. Eukaryotes possess these two forms, the former in mitochondria where the enzyme functions in energy generation and the latter in the cytoplasm where it functions in biosynthesis (Weitzman, 1982). The existence of NAD-linked isocitrate dehydrogenase activity is rare in bacteria although Streptococcus bovis (Burchall et al., 1964), Acetobacter suboxydans (Greenfield & Claus, 1969) and Thiobacillus thiooxidans (Matin & Rittenberg, 1971) possess solely this form. The existence of both enzymes has previously been reported in such disparate metabolic types as Acetobacter aceti (Greenfield & Claus, 1969), Xanthomonas pruni (Ragland et al., 1966), Rhizobium (Moustafa & Leong, 1975) and some methylotrophs and thiobacilli (Colby & Zatman, 1975; Matin & Rittenberg, 1971) but never in any member of the Rhodospirillaceae (Weitzman, 1982). The functional roles of these two enzymes, if two enzymes they are, is unclear. Tabita & Lundgren (1971) showed that Thiobacillus ferrooxidans produced NADP-linked isocitrate dehydrogenase under aerobic heterotrophic conditions and NAD-linked enzyme under anaerobic conditions where it functioned in biosynthesis. Such a situation has not been detected in any other bacterium nor is there a significant alteration in the activities of the two forms of the enzyme in R. vannielii under aerobic and anaerobic conditions

(D.J. Kelly, unpublished observations). The relative importance of these two enzymes in this organism clearly require further investigation.

The importance of the regulation of citrate synthase in the control of the TCA cycle was discussed above. Inhibition of activity by ATP was detected and has been previously reported in Rhodospirillum rubrum (Flechtner & Hanson, 1970) and Rhodobacter sphaeroides (Weitzman, 1982). The potential role of ATP inhibition when the TCA cycle is functioning in a bioenergetic role is clear but its importance in a biosynthetic cycle is puzzling. NADH inhibition was also observed and this has a clear potential functional role. However, Weitzman & Jones (1968) demonstrated a clear division in the citrate synthases of Gram-positive and Gram-negative bacteria with the former possessing citrate synthases of a low molecular weight that are insensitive to NADH inhibition, in contrast to the large, NADH-sensitive forms of the latter and thus the functional role of this feedback control is not as clear as it may at first appear. The reversal of citrate synthase inhibition by AMP was demonstrated in R. vannielii and has also been shown to occur in Rhodospirillum rubrum, Rhodobacter capsulatus and Rhodobacter sphaeroides (Weitzman, 1982). The absence of citrate synthase inhibition by 2-oxoglutarate in R. vannielii is in contradiction to the model of Weitzman & Dunmore (1969) who proposed that this represented a control mechanism in the incomplete TCA cycle, a hypothesis that was supported by such inhibition in facultatively anaerobic

bacilli (Tanaka & Hanson, 1975) and Clostridium acidurici (Gottschalk & Dittbrenner, 1970). These results and the observation of such control in the aerobic Bacillus subtilis (Flechtner & Hanson, 1969) must cast doubt on the hypothesis.

Albers & Gottschalk (1976) reported that R. vanniellii strain 7256 produced a fully functional glyoxylate shunt system but this could not be confirmed using strain Rm5. The distribution of glyoxylate shunt enzymes among the Rhodospirillaceae is somewhat variable. Rhodopseudomonas palustris, Rhodobacter capsulatus and Rhodocyclus tenuis have been shown to possess both malate synthase and isocitrate lyase (Kornberg & Lascelles, 1960; Albers & Gottschalk, 1976) but Rhodocyclus gelatinosus possesses isocitrate lyase activity only (Albers & Gottschalk, 1976) and Rhodobacter sphaeroides and Rhodospirillum rubrum possess malate synthase only (Kornberg & Lascelles, 1960; Albers & Gottschalk, 1976). It is this latter position that appears to exist in R. vanniellii Rm5 with, in common with most other species examined, stimulation of activity in cells grown on acetate (Eley et al., 1979; Albers & Gottschalk, 1976), although the physiological importance of this in an organism lacking a complete pathway is unclear.

The absence of NADH oxidase from autotrophic bacteria was at one time proposed as a cause of obligate autotrophy (Smith et al., 1967) but as assay methods improved this was shown to be untrue for cyanobacteria (Leach & Carr, 1968) and other autotrophic bacteria (Smith & Hoare, 1977). The absence of NADH oxidase activity from photoheterotrophically

grown cells of R. vannielii is not only surprising in this respect but is perplexing in that this is in distinct contrast to the other members of the Rhodospirillaceae so far examined (Beatty & Gest, 1981a; Cox et al., 1983). Indeed, Rhodobacter capsulatus not only possesses NADH oxidase activity under all growth conditions but activity does not alter with changing oxygen tension (Cox et al., 1983). The detection of activity under aerobic conditions and the presence of NADH dehydrogenase under all growth conditions (D.J. Kelly, unpublished observations) suggests that R. vannielii possesses a distinctly different electron transport system under anaerobic conditions from all other members of the Rhodospirillaceae so far examined.

The intermediary metabolism of R. vannielii examined in this study displayed a number of variations from that observed in other members of the Rhodospirillaceae. There remain a large number of unanswered questions with respect to the basic metabolic processes in this organism, particularly with regard to the activities of the two forms of isocitrate dehydrogenase detected and the biosynthetic systems operating in this organism. Furthermore, there remains a great deal to be ascertained regarding the activities and control of these enzymes in vivo. However, despite the unusual properties observed, there was no indication of any specialised adaptations for metabolism of organic carbon compounds present at very low concentrations although the very high activity of malate dehydrogenase may not be unrelated to this function if it were to occur in conditions containing

limiting concentrations of this substrate. Whilst this may reflect the composition of the medium necessary for producing the quantities of cells necessary for these studies, it is also possible that such adaptations may depend on equilibrium positions of enzyme catalysed reactions or will be unnecessary at such a stage of intermediary metabolism and will only be manifested at enzymes close to the first stages of substrate utilisation and capture.

3.3.6) Ribulose-1,5-bisphosphate carboxylase and
phosphoenolpyruvate carboxylase activities in
Rhodospirillum rubrum

If "first utilisation" enzyme reactions are those most likely to display adaptations for an oligotrophic mode of existence, then it is possible that these would be manifested most clearly at the level of CO₂ incorporation in

R. rubrum when growing under phototrophic conditions.

The two enzymes directly involved in this process are phosphoenolpyruvate (PEP) carboxylase and, most importantly, ribulose-1,5-bisphosphate carboxylase-oxygenase (RUBISCO).

The latter is the main enzyme involved in the incorporation of CO₂ in virtually all photoautotrophic organisms since it catalyses the key step in the Calvin cycle, whereas the former predominantly fulfills an anaplerotic role, except in some cyanobacteria where it appears to be the main route of CO₂ incorporation (Colman et al., 1976). The activity of RUBISCO in many bacteria is maximised under photoautotrophic conditions whereas in *R. rubrum* maximal

activity is observed in cells grown photoheterotrophically in malate or PM medium (Taylor & Dow, 1980). In this study, CO₂ incorporation by heterogeneous and swarmer cell populations was examined in whole cells and in direct assays of the two enzymes in cell-free extracts.

Fixation of CO₂ by whole cells proceeded at an equivalent rate in swarmer cells and heterogeneous populations incubated in the light and little incorporation was detected in the dark (Fig. 3.52). Mean detected CO₂ incorporation rates in heterogeneous and swarmer cell populations were 14.1 and 12.9 nmol min⁻¹ (mg protein)⁻¹ respectively for cells incubated in the light and 1.8 and 1.7 nmol min⁻¹ (mg protein)⁻¹ respectively for cells incubated in darkness.

PEP carboxylase activity in both cell types was detected at similar but low levels. In heterogeneous populations activity was 137.7 pmol CO₂ min⁻¹ (mg protein)⁻¹ and in swarmer cells 135.8 pmol CO₂ min⁻¹ (mg protein)⁻¹.

Activities of RUBISCO in cell-free extracts were also similar in heterogeneous and swarmer cell populations with mean detected CO₂ incorporation rates of 6.6 and 6.5 nmol min⁻¹ (mg protein)⁻¹ respectively. These activities are somewhat lower than those detected by Taylor & Dow (1980) but activity is highly dependent on growth conditions and phase. Inhibition of RUBISCO activity by 6-phosphogluconate (6-PGA) is, along with the control of the preceding enzyme in the cycle, phosphoribulokinase (Dijkhuizen & Harder, 1984), a presumed mechanism for the feedback control of the Calvin

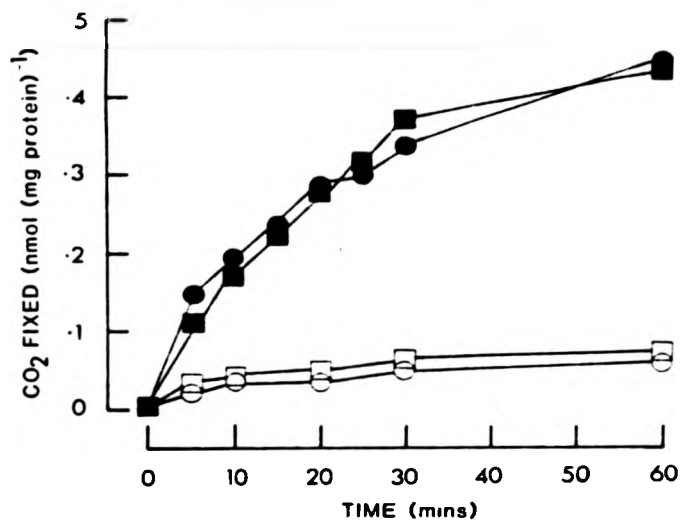


FIGURE 3.52 - CO₂ fixation by heterogeneous and synchronous swarmer cell populations of *Rhodospirillum rubrum*. For details of assay see text. (●) Heterogeneous population incubated in light. (○) Heterogeneous population incubated in dark. (■) Synchronous population incubated in light. (□) Synchronous population incubated in dark.

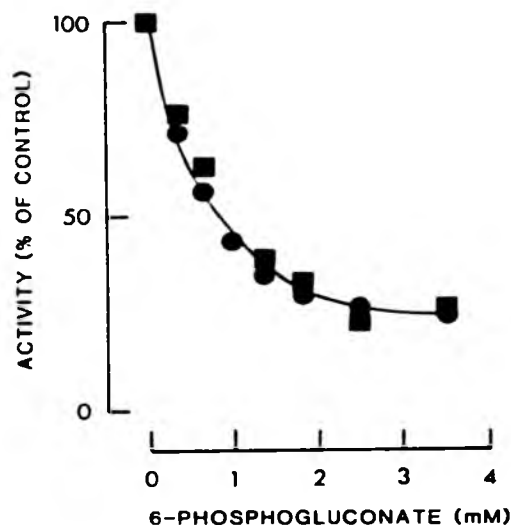


FIGURE 3.53 - Inhibition of *Rhodospirillum rubrum* RUBISCO activity by 6-phosphogluconate. Assays of RUBISCO activity were performed as described in the text using cell-free lysates from heterogeneous (●) and synchronous swarmer cell (■) populations in the presence of various concentrations of 6-phosphogluconate. Activity is expressed as a percentage of that occurring in the absence of inhibitor.

cycle and is a commonly observed feature of bacterial RUBISCO activity. Studies on the inhibition of RUBISCO activity in lysates from heterogeneous and swarmer cell populations by 6-PGA revealed there to be no significant difference between the two cell types (Fig. 3.53).

The control of CO₂ fixation in the Rhodospirillaceae has received widespread attention and has yielded a good deal of novel information. In plants and the vast majority of bacteria (Dijkhuizen & Harder, 1984), RUBISCO is composed of two subunits, one large and one small each present eight times (the structure is hence described as L₈S₈). However, Tabita & McFadden (1974a; 1974b) have shown that Rhodospirillum rubrum possesses a L₂ form and Gibson & Tabita (1977a; 1977b) have shown that Rhodobacter sphaeroides and Rhodobacter capsulatus possess both L₈S₈ and L₂ forms. In the latter cases the larger form of RUBISCO is 6-PGA-sensitive and the smaller form 6-PGA-insensitive. Sani (1985) has shown that Rhodopseudomonas blastica can synthesise both L₈S₈ and L₂ forms of RUBISCO and that under CO₂ limitation only the large form is synthesised. However, there is no evidence for the existence of a form of RUBISCO in R. vannielii other than its standard L₈S₈ (Taylor & Dow, 1980) nor was any difference detected in CO₂ fixing activity between cell types.

3.3.7) Differential expression of enzymes of intermediary metabolism in prosthecae and swarmer cells of prosthecae bacteria

This study has concentrated on the intermediary metabolism of Rhodospirillum rubrum for experimental reasons. No significant differences in enzyme activity or inhibitor sensitivity was detected among enzymes of the TCA cycle and related pathways nor was any difference detected in whole cell or cell-free extract CO_2 fixation. The latter was particularly surprising in view of the differential expression of the two forms of RUBISCO in a number of the Rhodospirillaceae, however, in these cases the control of expression appeared to be due to the relative abundance of CO_2 rather than to any inherent alterations in cell type and such investigations on R. rubrum should prove interesting.

It is clear that there are fundamental metabolic differences between the prosthecae and swarmer cells of the prosthecae bacteria studied to date (Dow et al., 1983) and the failure of this investigation to find any in the enzymes of intermediary metabolism associated with both anabolism and catabolism may be due to a variety of factors. Firstly, and most obviously, the wrong enzymes may have been chosen and more informative data may be obtained from other enzymes related to the capture, uptake and incorporation of substrates and the control of such factors as DNA transcription and rRNA synthesis which have been demonstrated to be limited or absent in the swarmer cell. It is also

possible that the regulation of enzymes of CO₂ fixation is manifested in a fashion different than that employed for enzymes of carbon incorporation under chemoheterotrophic conditions. Secondly, it is possible that the differences in activity lie at the in vivo level of feedback and other methods of control that cannot be modelled in crude assays of enzyme activity. Thirdly, it is possible that in order for the swarmer cell to rapidly adapt to an influx of nutrients in the natural environment it must possess a full complement of essential enzymes in order to avoid the competitive temporal disadvantage inherent in de novo synthesis of such components. Investigations of all three possibilities in a wide physiological variety of prosthecae bacteria are necessary if any major insight into the physiological quiescence of the swarmer cell are to be obtained.

3.4) OVERVIEW

This work has demonstrated that the prosthecae bacteria represent only a proportion of the bacterial flora of oligotrophic environments and that the present definitions of oligotrophy based on cultural parameters are unsound. Of particular importance to the prosthecae bacteria both in situ and in laboratory culture are the elongation of prosthecae and, particularly, the production of, apparently metabolically quiescent, swarmer cells as survival and dispersal stages. However, the physiological properties of this cell type remain unclear.

PART 4 - CONCLUSIONS

4.1) BACTERIA IN NATURAL LOW NUTRIENT ENVIRONMENTS

4.1.1) Population structure and dynamics

The observations made of bacterial populations occurring in low nutrient environments and in enrichment culture in this study serve to emphasise a number of points pertinent to any consideration of the bacterial flora of oligotrophic ecosystems. A variety of morphological types of organisms were observed, although observations suggested that a high surface area to volume ratio is a common adaptation in such situations. Interestingly, a high proportion of cells observed were flagellated and/or capable of accumulating nutrient reserve compounds. During continuous flow enrichment cultures, populations observed were highly dependent on flow rate. The populations produced at low flow rates were enriched in very small cells and in prosthecae cells which suggests that the latter were relatively uncompetitive at higher nutrient fluxes under the conditions employed.

These observations serve to confirm and underline the diversity of the microbial population in oligotrophic environments. It is unfortunate but understandable that the morphologically distinctive bacteria, particularly the prosthecae bacteria, have been overemphasised in a number of studies of the natural environment, occasionally to the almost total exclusion of the other components of the population. However, even the arbitrary divisions of bacteria into trophic groupings employed by Kuznetsov et al. (1979) resulted in the inclusion of representatives of many

morphologically indistinctive genera under the heading of oligotrophs. Furthermore, the majority of bacteria isolated as oligotrophs are rod-shaped or coccoid in morphology (e.g. Akagi et al., 1977; 1980; Hattori & Hattori, 1980; Yanagita et al., 1978). Whilst these data may well represent, in part, products of the artificial conditions employed for isolation, particularly if lysis of delicate oligotrophic cells occurs under such conditions, they stress once again the variety of organisms present in significant numbers in oligotrophic environments and that the prosthecae bacteria are by no means the only forms adapted for growth and survival under conditions of extreme nutrient limitation.

The basic observations made in this study also indicate the importance of surface-associated growth in the aquatic low nutrient environment. In continuous flow enrichment culture the bulk of the population was observed at surfaces at the lowest medium flow rates and the majority of bacteria observed were apparently capable of surface attachment. It is clear that those studies which consider the environment as being nutritionally homogeneous are naive in the extreme. As Fletcher & Marshall (1982) have reviewed, the variety of interfaces in the natural environment is great and the potential advantages of being surface-associated clear. It has been shown (Simon, 1985) that the process of bacterial adhesion to surfaces is complicated beyond simple considerations of surface area and nutrient status by the fact that in the natural environment there is a preferential colonisation of organic particles over inorganic ones.

Furthermore, it is not only those bacteria capable of efficient attachment to surfaces that benefit from the localised concentration of nutrients at surfaces, for example Hermansson & Marshall (1985) have demonstrated that surface-localised substrates can be exploited by non-adhesive marine bacteria even in the presence of attached competing cells. In the light of these observations and those demonstrating increased attachment potential during nutrient starvation (Kjelleberg & Dahlbäck, 1984; Humphrey & Marshall, 1984), it is evident that the differentiation of bacteria into groupings of obligate and facultative oligotrophs on the basis of their ability or inability to attach to surfaces (e.g. Ishida *et al.*, 1980; Horowitz *et al.*, 1983; Yanagita *et al.*, 1978) is highly questionable. It is apparent that there exists a need for detailed examinations to be made of the physical and biological nature of oligotrophic environments and that until such information is available the relative activities of the bacteria therein will remain a matter for debate.

4.1.2) Adaptations of prosthecate bacteria to nutrient limitation

Having emphasised above the variety of bacteria in the low nutrient environment and the occasional overestimation of the importance of the prosthecate bacteria, it is important to correct the balance and discuss the latter organisms in detail. Whilst there exist a number of prosthecate bacteria which are highly competitive in certain high nutrient

conditions, it is evident that the proportion of prosthecae bacteria in most environments is inversely proportional to the nutrient level in that environment and, therefore, that the prosthecae bacteria can represent, at least to an extent, model oligotrophic organisms. This designation in no way implies that they represent forms more highly specialised than morphologically "typical" organisms in the same environment but merely that, by virtue of their morphologies and life cycles, they represent a means of unequivocally studying two of the factors believed to be of particular importance in ensuring competitiveness in the oligotrophic environment, increased surface area to volume ratio and the production of dormant and/or dispersal cell types.

The elongation of prosthecae in response to decreasing nutrient concentrations was demonstrated in this study in both batch and continuous culture and was confirmatory of much previous data (e.g. Schmidt & Stanier, 1966; Whittenbury & Dow, 1977; Poindexter, 1984a; 1984b). However, there was no evidence to support the model of Poindexter (1984a; 1984b) that assigned prosthecae to a specialised role in phosphate uptake alone. Elongation of prosthecae in response to both carbon and phosphate limitation was observed and the elongation of prosthecae in response to increased medium calcium levels was not observed at the concentrations employed. It can be argued that the conditions employed by Poindexter (1984a; 1984b) were more extreme than those employed in this study, involving as they did the use of far higher concentrations of medium constituents but conversely

such high concentrations are unlikely to have much relevance to most of the natural environment. Increase in cell surface area to volume ratio clearly occurs with increasing nutrient stress but it would be incorrect to assign this behaviour to the concentration of one nutrient alone. It appears likely that the elongation of prosthecae is a function of the balance of nutrients available to the cells or, more probably, to the actual growth rate of those cells.

These observations beg the question as to the functions of prosthecae. It appears probable that, in the light of their reappearance in groups of distinct evolutionary origin, the answer is several. Their role in increasing surface area to volume ratio is clear if, as yet, unquantified and they appear to have an equivalent function in certain photosynthetic prosthecate bacteria. In those forms which reproduce by budding at the distal ends of their prosthecae they may additionally function for physical separation of mother and daughter cell. In those forms which attach by means of holdfasts at the ends of their prosthecae they may serve as a means of raising the main cell body away from the other organisms present at a surface which may confer an advantage in the competition for nutrients and/or oxygen or may, as is the case in such forms as Pedomicrobium, function to aid the dispersal of the organism by ensuring the daughter cell is released away from the surface. The increased buoyancy of prosthecate cells also suggests that prosthecae may function as aids to flotation.

It has been proposed that the production of swarmer

cells or dormant cell types plays a major part in the survival of bacteria in the oligotrophic environment (Dow et al., 1983; Stevenson, 1978) and can readily be studied in prosthecate bacteria. In this investigation it was shown that accumulation of swarmer cells occurred with increasing nutrient stress and was largely independent of the identity of the limiting nutrient. Evidence is amassing to suggest that swarmer-type cells exist in many bacteria growing under conditions of nutrient stress (Dow et al., 1983; Morgan & Dow, 1985) and the prosthecate bacteria offer the simplest means of studying this cell type. It is unfortunate that little is known concerning the maintenance of and exit from the swarmer cell state. It is clear that investigations into these, apparently not so unique, cell types should yield information that will be relevant not only to our understanding of these organisms themselves but will have significance to most bacteria growing under more natural conditions than those normally employed in laboratory culture.

4.2) OLIGOTROPHY - HOW VALID A CONCEPT?

4.2.1) Ecological considerations

The definitions of the terms oligotrophic and copiotrophic as currently applied by the majority of microbiologists rely, at best, on a quantitative definition of the environment or organism in terms of organic carbon or, at worst, on the cultivation of bacteria under highly artificial conditions.

Setting aside the absolute definitions employed, it is clear that such subdivisions in no way represent the situation that is observed in the natural environment. It is evident that growth limitation in the natural environment is dependent not only on absolute levels of organic carbon but on a variety of other factors, nutritional and physical (Morgan & Dow, 1986). Furthermore, as discussed above, the presently employed definitions of oligotrophy with respect to the natural environment either totally neglect interfaces or apply them as criteria with which different trophic types can be separated. It is becoming increasingly clear that the natural low nutrient environment cannot be classified into arbitrary groupings in such a way.

It is also becoming evident that mere observations on and isolations from the natural environment in no way illustrate the patterns of bacterial activity in situ (Morita, 1982). The existence of dormant or swarmer-type cells in the natural environment is now becoming more evident, and has been demonstrated to be important in continuous culture in this study, and it is clear that only a proportion of bacterial cells in a given environment are active in any given period of time. This temporal consideration applicable to the natural environment has, as yet, received little attention. It is clear that there exist vast volumes of the biosphere that are characterised by an extremely low flux of nutrients averaged over space and time and that there exist bacteria in such environments that are highly competitive under such conditions. However, it is now

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becoming apparent that the presently accepted definitions of oligotrophy are far from adequate for classifying natural environments and the organisms therein.

4.2.2) Physiology of oligotrophic bacteria

The concept of model oligotrophic bacteria (Hirsch, 1979; Poindexter, 1981a) included the assignation of a number of organisms to this grouping, most notably the budding, prosthecae bacteria. The model oligotrophs were proposed to possess a variety of distinct physiological properties. The organism studied in this investigation, R. vannielii, apparently did not possess any specific adaptations to an oligotrophic mode of existence as detected by assays of the enzymes of the TCA cycle or CO₂ fixation. Furthermore, no significant differences were detected between the prosthecae and swarmer cells of this organism. However, the swarmer cells of prosthecae bacteria represents an adaptation for the "tuning" of a daughter cell to new, potentially hostile environmental conditions. By producing a cell type that inherits little environmentally controlled physiological "information" from the mother cell the bacteria are in a position to produce cells with an optimal survival capacity in a continually changing environment. Indeed, many bacteria can respond to changing nutrient conditions not only by the induction of catabolic enzyme systems and nutrient uptake systems when a novel nutrient is introduced but by altering the metabolic pathway employed for the utilisation of a given nutrient at different concentrations. For example, Neijssel

et al. (1975) demonstrated that Klebsiella aerogenes, an organism not thought of as being oligotrophic, utilised a low affinity glycerol dehydrogenase for the assimilation of glycerol at high dilution rates in continuous culture and a high affinity glycerol kinase at low dilution rates. A close coupling of glycerol uptake and respiration was also noted which may potentially function as a means of minimising efflux of nutrients accumulated from a nutrient-poor environment. Similar phenomena, characteristic of model oligotrophs (Hirsch, 1979), have also been detected in organisms not thought of as oligotrophic, for example the utilisation of multiple substrates by Pseudomonas oxalaticus (Harder & Dijkhuizen, 1983), the hoarding of transported carbon sources by constitutive strains of E. coli and the metabolic repression observed in the same organism (Koch, 1979). It is also unlikely that all chemoheterotrophic bacteria successfully adapted for the low nutrient environment are obligate aerobes (Poindexter, 1981b), particularly since a number of oligotrophic isolates are capable of growth under anaerobic conditions (e.g. Seliberia-like strains; Old & Wong, 1972) and a large volume of the oligotrophic environment may be anoxic, particularly in deep oceanic waters.

It is apparent that the proposed physiological adaptations for oligotrophic growth are possessed by all bacteria examined to date (Morgan & Dow, 1986) and, therefore, that such adaptations in no way represent specific properties of "unusual" bacteria to extreme environments. In

addition, the properties that have been proposed as possibly accounting for obligate oligotrophy in bacteria, are themselves questionable. Kuznetsov et al., (1979) suggested that the inability of oligotrophic isolates to grow at higher growth rates was due to them producing hydrogen peroxide which, due to their lack of catalase, they were unable to detoxify. However, many of those bacteria which behave as oligotrophs on first isolation possess catalase (e.g. Zobell & Upham, 1944), as do many "model" oligotrophs, for example Caulobacter and Hyphomicrobium (Poindexter, 1964; Harder & Attwood, 1978). Investigations into the adaptations of bacteria to nutrient limitation and changes in nutrient concentrations in laboratory culture are therefore imperative, if an understanding of the responses of all bacteria to nutrient limitation in the natural environment is to be reached. Until this is understood in more detail it is unlikely that the physiological properties enabling bacteria to grow in extremely low nutrient environments can be ascertained. In the latter case it will be particularly interesting to discover whether the adaptations are indeed specific or merely represent a conglomeration of those occurring in all bacteria. In the light of the investigations performed in this study and those data previously published, the question therefore arises as to the existence of the proposed trophic groupings of bacteria.

4.2.3) Obligate, facultative and model oligotrophs

As has been discussed above, there is a significant amount of

evidence to suggest that the currently accepted definitions of oligotrophy and copiotrophy are inadequate in both physiological and ecological terms. However, there is also evidence that the definitions employed by Kuznetsov et al. (1979) and subsequent workers are themselves flawed. Martin & Macleod (1984) demonstrated that manifestations of oligotrophy in marine bacteria in laboratory culture are dependent upon the carbon source employed in the experiments and these observations were confirmed in this investigation using fresh water isolates. Furthermore, Bell (1984) showed that the populations developing in a system employing natural algal exudates as the sole sources of nutrients were markedly different than those developing when man-made substrates were employed. Thus the distinction between facultative and obligate oligotrophs and copiotrophs appears even more mistaken, since it appears that the evidence on which the divisions are made is itself faulty. This opinion is supported by observations of "substrate accelerated death" in microorganisms: bacteria grown under extreme carbon-limitation display a marked reduction in viability when organic carbon is subsequently supplied (Strange & Dark, 1965; Straskrbová, 1983).

Since these basic distinctions are themselves inherently flawed, is there a need to invoke the existence of model oligotrophs? In addition to the physiological evidence against the existence of such organisms currently maintained in culture, there is the question of the evolutionary competitiveness of such an organism. Koch (1979) proposed

that there are theoretically two types of oligotroph that could be selected for in continuous culture, one that is capable of utilising substrates present continuously at extremely low concentrations and one that can utilise influxes of low concentrations of nutrients at random periods, interspersed by periods of total nutrient deprivation. Obviously, these two organisms would differ greatly from one another and each would be uncompetitive in the environment used for the selection of the other. All real organisms, however, embody attributes of both extreme forms and many are apparently able to alter their responses in the light of changing environmental conditions. It is evident that each adaptation employed by an organism in the natural environment has been optimised by natural selection and that these organisms must be able to respond to any environmental change in the future. Consequently, there will be no model oligotroph, since that organism would be potentially equipping itself for evolutionary failure with a change in environmental conditions (Koch, 1979). These considerations, in conjunction with a logical consideration of the nature of the natural environment which is subject to constant change of all parameters, make it unlikely that there exist model or obligate oligotrophs of the types presently envisaged.

4.3) FINAL THOUGHTS

The ubiquity of low nutrient environments is undeniable and, by virtue of their sheer volume, their importance in the

global cycling of nutrients cannot be overemphasised. Indeed, it is clear that the majority of bacteria in their natural environments must undergo periods of "feast and famine" (Koch, 1971) with the latter frequently far longer than the former and, in the environment commonly classified as oligotrophic, with the former being less of a feast and more of a mere taste of nutrition. It is gradually being appreciated that the properties of bacteria under such conditions may be vastly different from those observed in normal laboratory culture and that this not only has academic interest but is a vital consideration if we are to understand such phenomena as the effects of xenobiotics on the natural environment and the survival of plant and animal pathogens. The latter has recently been clearly demonstrated by the observation that Legionella pneumophila, when grown under conditions approximating those occurring in the natural environment, possesses properties markedly different from those observed in batch culture, including its sensitivity to antimicrobial agents (Berg et al., 1985). Such observations will undoubtedly become more frequent.

The criticisms made of the terms applied to low nutrient environments and the organisms therein by no means negates the fact that there exist bacteria which are particularly competitive under such conditions. It is, however, important to recognise that all bacteria can adapt their physiology and, frequently, morphology in order to maximise survival and competitive potential under relative nutrient limitation. Those bacteria competitive under

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oligotrophic conditions in no way represent unusual organisms but merely represent the end products of selection for adaptations that increase nutrient capture and utilisation efficiency but do not render the organism potentially uncompetitive when environmental conditions change to a degree characteristic of the majority of microbial habitats unaltered by man.

This investigation has demonstrated the existence of a wide variety of bacteria in the low nutrient fresh water environment which includes prosthecae bacteria but these in no way exclude other bacteria. The potential importance of increased surface area to volume ratio and the production of swarmer cells in ensuring the competitiveness of these organisms has also been demonstrated. It is exciting to realise the potential importance of swarmer-type cells in other groups of bacteria found under conditions of nutrient stress, i.e. virtually all bacteria. The prosthecae bacteria therefore represent excellent systems in which all aspects of these cells can be investigated and we may be some way towards confirming the proposal of Koch (1979):

"survival mechanisms during the transit of a propagule to new habitat should be an important part of the biology of any inhabitant of an extreme environment even if studies to date have not uncovered any mechanisms such as, or akin to, sporulation."

PART 5 - REFERENCES

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APPENDIX 1 - LIST OF PUBLICATIONS

ACCEPTED FOR PUBLICATION

P. Morgan & C.S. Dow (1985) "Environmental control of cell type expression in prosthecate bacteria". In: "Bacteria in Their Natural Environments" (M. Fletcher & G.D. Floodgate, eds.), pp 131-169. Academic Press, London.

P. Morgan & C.S. Dow (1986) "Bacterial adaptations for growth in low nutrient environments". In: "Microbes in Extreme Environments" (R.A. Herbert & G.A. Codd, eds.), in press. Academic Press, London.

SUBMITTED FOR PUBLICATION

P. Morgan, D.J. Kelly & C.S. Dow "The tricarboxylic acid cycle of heterogeneous and swarmer cell populations of Rhodomicrobium vannielii Rm5". Journal of General Microbiology.

P. Morgan & C.S. Dow "Effects of organic carbon concentration on growth yields of 'model oligotrophic' and newly isolated fresh water 'oligotrophic' bacteria". FEMS Letters.

P. Morgan & C.S. Dow "The effects of nutrient concentration on cell type expression and cell morphology of Hyphomicrobium X". Journal of General Microbiology.